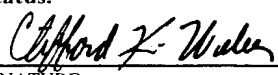


FORM PTO-1390 (REV. 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER THA01-C1003	
<b>TRANSMITTAL LETTER TO THE UNITED STATES          DESIGNATED/ELECTED OFFICE (DO/EO/US)          CONCERNING A FILING UNDER 35 U.S.C. 371</b>				U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>09/763777</b>	
INTERNATIONAL APPLICATION NO. PCT/US99/19011		INTERNATIONAL FILING DATE August 17, 1999		PRIORITY DATE CLAIMED August 17, 1998	
TITLE OF INVENTION Imaging with TC-99M labeled Fibrin-Alpha-Chain Peptide					
APPLICANT(S) FOR DO/EO/US Thomas Jefferson University					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1. <input type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below. 4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> has been communicated by the International Bureau. c. <input checked="" type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). a. <input type="checkbox"/> is attached hereto. b. <input checked="" type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).					
<b>Items 11 to 20 below concern document(s) or information included:</b>					
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input type="checkbox"/> A FIRST preliminary amendment. 14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input checked="" type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825. 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 20. <input type="checkbox"/> Other items or information:					

INTERNATIONAL APPLICATION NO. PCT/US99/19011				ATTORNEY'S DOCKET NUMBER THA01-C1003	
21: <input checked="" type="checkbox"/> The following fees are submitted: <b>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):</b> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... <b>\$1000.00</b> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <b>\$860.00</b> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$710.00</b> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$690.00</b> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... <b>\$100.00</b> <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				<b>CALCULATIONS PTO USE ONLY</b>          \$ 690.00	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$	
Total claims	9 - 20 =	0	x <b>\$18.00</b>	\$	
Independent claims	2 - 3 =	0	x <b>\$80.00</b>	\$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ <b>\$270.00</b>	\$	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				+ \$ 345.00	
<b>SUBTOTAL =</b>				\$ 345.00	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
<b>TOTAL NATIONAL FEE =</b>				\$ 345.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property +				\$	
<b>TOTAL FEES ENCLOSED =</b>				\$ 345.00	
				Amount to be refunded:	\$
				charged:	\$
a. <input type="checkbox"/> A check in the amount of \$ _____ to cover the above fees is enclosed. b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. <u>50-0491</u> in the amount of \$ <u>345.00</u> to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>50-0491</u> . A duplicate copy of this sheet is enclosed. d. <input type="checkbox"/> Fees are to be charged to a credit card. <b>WARNING:</b> Information on this form may become public. <b>Credit card</b> <b>information should not be included on this form.</b> Provide credit card information and authorization on PTO-2038.					
<b>NOTE:</b> Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO:					
 SIGNATURE Clifford K. Weber NAME 42,215 REGISTRATION NUMBER					

Serial No. 09/763,777  
Group Art Unit No.  
Docket No. THA01.C1003

09/763,777 09/763,777  
04 June 2002

**PRELIMINARY AMENDMENT**

Applicant respectfully requests entrance of the following Preliminary Amendment into the record of the above-captioned application.

**IN THE BACKGROUND OF THE INVENTION:**

At page 3, line 25, please delete "H-Gly-Pro-Arg-OH" and insert --H-Gly-Pro-Arg-OH (SEQ ID NO:1)-- in place thereof.

At page 3, line 30, please delete "H-Gly-Pro-Arg-Pro-OH" and insert --H-Gly-Pro-Arg-Pro-OH (SEQ ID NO:2)-- in place thereof.

At page 3, line 33, please delete "H-Gly-Pro-Arg-Pro-Pro-OH" and insert -- H-Gly-Pro-Arg-Pro-Pro-OH (SEQ ID NO:3)-- in place thereof.

**IN THE DEFINITIONS:**

At page 4, line 14, please delete "Gly-Pro-Arg-Pro-Pro-Ana-Gly-Gly-(D)-Ala-Gly" and insert --Gly-Pro-Arg-Pro-Pro-Aba-Gly-Gly-(D)-Ala-Gly-- in place thereof.

At page 4, line 15, please delete "(SEQ ID NO:1)" and insert --(SEQ ID NO:5)-- in place thereof.

**IN THE DETAILED DESCRIPTION:**

At page 6, line 19, please delete "Gly-(D)-Ala-Gly-Gly-" and insert --Gly-(D)-Ala-Gly-Gly (SEQ ID NO:4)-- in place thereof.

At page 6, line 24, please delete "(4-aminobutyric acid)" and insert --((D)-4-aminobutyric acid)-- in place thereof.

At page 6, line 30, please delete "Gly-Pro-Arg-Pro-Pro-Aba-Gly-Gly-(D) Ala-Gly" and insert --Gly-Pro-Arg-Pro-Pro-Aba-Gly-Gly-(D) Ala-Gly (SEQ ID NO:5)-- in place thereof.

At page 17, line 23, please delete "H-Gly-Pro-Arg-OH" and insert --H-Gly-Pro-Arg-OH (SEQ ID NO:1)-- in place thereof.

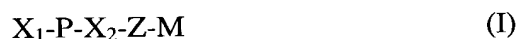
Serial No. 09/763,777  
Group Art Unit No.  
Docket No. THA01.C1003

At page 17, line 26, please delete "H-Gly-Pro-Arg-Pro-OH" and insert --H-Gly-Pro-Arg-Pro-OH (SEQ ID NO:2)-- in place thereof.

At page 17, line 31, please delete "H-Gly-Pro-Arg-Pro-Pro-OH" and insert -- H-Gly-Pro-Arg-Pro-Pro-OH (SEQ ID NO:3)-- in place thereof.

**IN THE CLAIMS:**

Claim 1 (Amended). A composition having formula I or II:



wherein:

X<sub>1</sub> is from zero to twenty natural or synthetic amino acids;

P is a peptide comprising Gly Pro Arg (SEQ ID NO: [2] 1), or an analog or fragment thereof;

X<sub>2</sub> is from zero to twenty natural or synthetic amino acids;

Z is a linker comprising one or more natural or synthetic amino acids; and

M is a radiolabeling moiety comprised of a chelating moiety capable of complexing with a selected radionuclide.

Claim 6 (Amended). The composition according to Claim 1, wherein M comprises Gly -(D)-Ala-Gly-Gly (SEQ ID NO: [3] 4) as a chelating moiety for a radionuclide.

These amendments to the specification and claims are merely clarifications or corrections of obvious typographical errors. No new matter is added by these amendments.

Serial No. 09/763,777  
Group Art Unit No.  
Docket No. THA01.C1003

**STATEMENT PURSUANT TO 37 C.F.R. 1.821(f) and (g)**

Applicant hereby states that the enclosed substitute CRF diskette and paper copy of the "Sequence Listing" submitted herewith are identical and contain no new matter. 37 C.F.R. 1.821(f) and 1.821(g).

**REMARKS**

Please charge any fee deficiency required by this paper or credit any amount paid in excess to Account 50-0491.

Respectfully submitted,

Madhukar (Mathew) L. Thakur

6/4/2001  
Date

BY:

Clifford K. Weber, Esq.  
Clifford K. Weber, Esq.  
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J:\CWEBER\THA01.NP003\RESPONSE TO SEQUENCE REQUIRMENTS

Serial No. 09/763,777  
Group Art Unit No.  
Docket No. THA01.C1003

\* \* \*

CERTIFICATE OF MAILING UNDER 37 C.F.R. 1.10

EXPRESS MAIL Mailing Label Number: **EJ 464 024 164 US**

Date of Deposit: June 4, 2001

I hereby certify that this paper and/or fee is being deposited with the United States Postal Service, "**EXPRESS MAIL - POST OFFICE TO ADDRESSEE**" service under 37 C.F.R. 1.10, on the date indicated above, and is addressed to Box Patent Application, Commissioner of Patents & Trademarks, Washington, D.C., 20231.

Clifford Kent Weber  
(Type or print name of person mailing paper.)

*Clifford Kent Weber*  
(Signature of person mailing paper)



## UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents, Box PCT  
United States Patent and Trademark Office  
Washington, D.C. 20231  
www.uspto.gov

U.S. APPLICATION NO.		FIRST NAMED APPLICANT	ATTY. DOCKET NO.
09/763777		THAKUR	THA01-C1003
INTERNATIONAL APPLICATION NO.			
PCT/US99/19011			

I.A. FILING DATE	PRIORITY DATE
17 AUG 99	17 AUG 98

DATE MAILED: 03 APR 2001

CLIFFORD K WEBER  
THOMAS JEFFERSON UNIVERSITY  
44 LAKEVIEW DRIVE  
CHERRY HILL, NJ 08003

## NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

1. The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as ☐ a Designated Office (37 CFR 1.494) ☒ an Elected Office (37 CFR 1.495):

- |  |   |
|--|---|
| <input checked="" type="checkbox"/> U.S. Basic National Fee.   | <input type="checkbox"/> Indication of Small Entity Status.                         |
| <input checked="" type="checkbox"/> Copy of the international application.   | <input type="checkbox"/> Translation of the international application into English. |
| <input checked="" type="checkbox"/> Oath or Declaration of inventors(s).   | <input type="checkbox"/> Translation of Article 19 amendments into English.         |
| <input type="checkbox"/> Copy of Article 19 amendments.  | <input type="checkbox"/> Other:   |
| <input type="checkbox"/> Priority Document.  |   |
| <input checked="" type="checkbox"/> The International Preliminary Examination Report in English and its Annexes, if any. |   |
| <input type="checkbox"/> Translation of Annexes to the International Preliminary Examination Report into English.        |   |

2. ☐ Applicant has requested early processing under 35 U.S.C. 371(f) but has not filed the following indicated items and/or the indicated items in paragraph 3 below. The Basic National Fee and the copy of the international application must be filed prior to 20 or 30 months from the priority date to avoid abandonment.

- ☐ U.S. Basic National Fee. ☐ Copy of the international application.

3. The following items **MUST** be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

- ☐ a. Translation of the application into English. A processing fee will be required if submitted later than the appropriate 20 or 30 months from the priority date.
- ☐ The current translation is defective for the reasons indicated on the attached Notice of Defective Translation.
- ☐ b. Processing fee for providing the translation of the application and/or the Annexes later than the appropriate 20 or 30 months from the priority date (37 CFR 1.492(f)).
- ☐ c. Oath or declaration of the inventors, in compliance with 37 CFR 1.497(a) and (b), properly identifying the application (preferably by the International application number and international filing date). A surcharge will be required if submitted later than the appropriate 20 or 30 months from the priority date.
- ☐ The current oath or declaration does not comply with 37 CFR 1.497(a) and (b) for the reasons indicated on the attached PCT/DO/EO/917.
- ☐ d. Surcharge for providing the oath of declaration later than the appropriate 20 or 30 months from the priority date (37 CFR 1.492(e)).

4. Additional claim fees of \$\_\_\_\_\_ as a ☐ large entity ☐ small entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due (37 CFR 1.492(g)). See attached PTO-875.

5. ☒ Applicant has not submitted the required sequence listing pursuant to 37 CFR 1.821-1.825. See attached PCT/DO/EO/920.

**ALL OF THE ITEMS SET FORTH IN 3(a)-3(d), 4 AND 5 ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTHS FROM THE DATE OF THIS NOTICE OR BY 22 OR 32 MONTHS (where 37 CFR 1.495 applies) FROM THE PRIORITY DATE FOR THE APPLICATION, WHICHEVER IS LATER. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.**

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

6. If box 3a or 3c is checked, a translation of the Annexes **MUST** be submitted no later than the time period set above or the Annexes will be cancelled. A processing fee will be required if submitted later than 20 or 30 months from the priority date.

7. ☐ The Article 19 amendments are cancelled since a translation was not provided by the appropriate 20 (37 CFR 1.494(d)) or 30 (37 CFR 1.495(d)) months from the priority date.

Applicant is reminded that any communication to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above. (37 CFR 1.5)

**A copy of this notice MUST be returned with this response.**

Enclosed: ☐ PCT/DO/EO/917  
☐ PTO-875

☐ Notice of Defective Translation  
☒ PCT/DO/EO/920

Pat Booker, Paralegal



## UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents, Box PCT  
United States Patent and Trademark Office  
Washington, D.C. 20231  
www.uspto.gov

U.S. APPLICATION NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
09/763777	THAKUR	M
CLIFFORD K WEBER THOMAS JEFFERSON UNIVERSITY 44 LAKEVIEW DRIVE CHERRY HILL, NJ 08003		THA01-C1003
INTERNATIONAL APPLICATION NO.		
PCT/US99/19011		
I.A. FILING DATE		PRIORITY DATE
17 AUG 99		17 AUG 98
DATE MAILED: 03 APR 2001		

**NOTIFICATION TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS  
CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE  
DISCLOSURES**

Applicant has submitted papers under 35 U.S.C. 371 to enter the national stage in the United States of America. The items indicated below, however, are missing. The period within which to correct the deficiency noted below and avoid abandonment is set forth in the accompanying Notification.

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 CFR 1.821-1.825 for the following reason(s):

- ☒ The application fails to comply with the requirements of 37 CFR 1.821-1.825.
- ☐ This application does not contain, a "Sequence Listing" as a separate part of the disclosure on paper copy or compact disc, as required by 37 CFR 1.821(c).
- ☐ A copy of the "Sequence Listing" in computer readable format has not been submitted as required by 37 CFR 1.821(e).
- ☒ A copy of the "Sequence Listing" in computer readable form has been submitted. The content of the computer readable form, however, does not comply with the requirements of 37 CFR 1.822 and/or 1.832, as indicated on the attached marked-up copy of the "Raw Sequence Listing".
- ☐ The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A substitute computer readable form must be submitted as required by 37 CFR 1.825(d).
- ☐ The paper copy or compact disc of the "Sequence Listing" is not the same as the computer readable form of the "Sequence Listing" as required by 37 CFR 1.821(e).
- ☒ Other: See attachment

**APPLICANT MUST PROVIDE:**

- ☐ An initial or substitute computer readable form (CRF) of the "Sequence Listing."
- ☐ An initial or substitute paper copy or compact disc of the "Sequence Listing," as well as an amendment directing its entry into the specification.
- ☐ A statement that the contents of the paper or compact disc and the computer readable form are the same and, where applicable, include no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b) or 1.825(d).

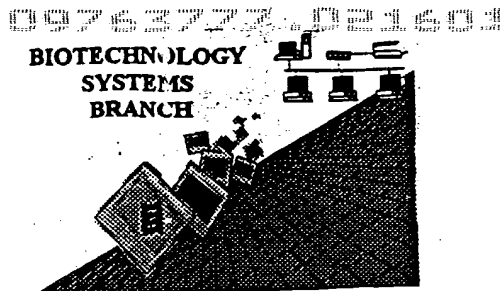
**FOR QUESTIONS REGARDING COMPLIANCE WITH THESE REQUIREMENTS, PLEASE  
CALL:**

(703) 308-4216, for Rules interpretation,  
(703) 308-4212, for CRF submission help,  
(703) 287-0200, for PatentIn software help.

Pat Booker, Paralegal

Telephone: 703-305-3738





## **RAW SEQUENCE LISTING ERROR REPORT**

The Biotechnology Systems Branch of the Scientific and Technical Information Center (STIC) detected errors when processing the following computer readable form:

Application Serial Number: 09/763,777  
Source: PERO9  
Date Processed by STIC: 3/7/2001

**THE ATTACHED PRINTOUT EXPLAINS DETECTED ERRORS.**

**PLEASE FORWARD THIS INFORMATION TO THE APPLICANT BY EITHER:**

- 1) INCLUDING A COPY OF THIS PRINTOUT IN YOUR NEXT COMMUNICATION TO THE APPLICANT, WITH A NOTICE TO COMPLY or,**
- 2) TELEPHONING APPLICANT AND FAXING A COPY OF THIS PRINTOUT, WITH A NOTICE TO COMPLY**

**FOR CRF SUBMISSION QUESTIONS, PLEASE CONTACT MARK SPENCER, 703-308-4212.**

**FOR SEQUENCE RULES INTERPRETATION, PLEASE CONTACT ROBERT WAX, 703-308-4216.**

**PATENTIN 2.1 e-mail help: [patin21help@uspto.gov](mailto:patin21help@uspto.gov) or phone 703-306-4119 (R. Wax)**

**PATENTIN 3.0 e-mail help: [patin3help@uspto.gov](mailto:patin3help@uspto.gov) or phone 703-306-4119 (R. Wax)**

**TO REDUCE ERRORED SEQUENCE LISTINGS, PLEASE USE THE CHECKER VERSION 3.0 PROGRAM, ACCESSIBLE THROUGH THE U.S. PATENT AND TRADEMARK OFFICE WEBSITE. SEE BELOW:**

### **Checker Version 3.0**

The Checker Version 3.0 application is a state-of-the-art Windows based software program employing a logical and intuitive user-interface to check whether a sequence listing is in compliance with format and content rules. Checker Version 3.0 works for sequence listings generated for the original version of 37 CFR §§1.821 - 1.825 effective October 1, 1990 (old rules) and the revised version (new rules) effective July 1, 1998 as well as World Intellectual Property Organization (WIPO) Standard ST.25.

Checker Version 3.0 replaces the previous DOS-based version of Checker, and is Y2K-compliant. Checker allows public users to check sequence listings in Computer Readable form (CRF) before submitting them to the United States Patent and Trademark Office (USPTO). Use of Checker prior to filing the sequence listing is expected to result in fewer errored sequence listings, thus saving time and money.

**Checker Version 3.0 can be down loaded from the USPTO website at the following address:**

**<http://www.uspto.gov/web/offices/pac/checker>**

# Raw Sequence Listing Error Summary

## ERROR DETECTED SUGGESTED CORRECTION

SERIAL NUMBER: 09/763,777

ATTN: NEW RULES CASES: PLEASE DISREGARD ENGLISH "ALPHA" HEADERS, WHICH WERE INSERTED BY PTO SOFTWARE

- 1        Wrapped Nucleics The number/text at the end of each line "wrapped" down to the next line.  
This may occur if your file was retrieved in a word processor after creating it.  
Please adjust your right margin to .3, as this will prevent "wrapping".
- 2        Wrapped Aminos The amino acid number/text at the end of each line "wrapped" down to the next line.  
This may occur if your file was retrieved in a word processor after creating it.  
Please adjust your right margin to .3, as this will prevent "wrapping".
- 3        Incorrect Line Length The rules require that a line not exceed 72 characters in length. This includes spaces.
- 4        Misaligned Amino Acid Numbering The numbering under each 5th amino acid is misaligned. This may be caused by the use of tabs between the numbering. It is recommended to delete any tabs and use spacing between the numbers.
- 5        Non-ASCII This file was not saved in ASCII (DOS) text, as required by the Sequence Rules.  
Please ensure your subsequent submission is saved in ASCII text so that it can be processed.
- 6        Variable Length Sequence(s)        contain n's or Xaa's which represented more than one residue.  
As per the rules, each n or Xaa can only represent a single residue.  
Please present the maximum number of each residue having variable length and indicate in the (ix) feature section that some may be missing.
- 7        PatentIn ver. 2.0 "bug" A "bug" in PatentIn version 2.0 has caused the <220>-<223> section to be missing from amino acid sequence(s)       . Normally, PatentIn would automatically generate this section from the previously coded nucleic acid sequence. Please manually copy the relevant <220>-<223> section to the subsequent amino acid sequence. This applies primarily to the mandatory <220>-<223> sections for Artificial or Unknown sequences.
- 8        Skipped Sequences (OLD RULES) Sequence(s)        missing. If intentional, please use the following format for each skipped sequence:  
(2) INFORMATION FOR SEQ ID NO:X:  
(i) SEQUENCE CHARACTERISTICS:(Do not insert any headings under "SEQUENCE CHARACTERISTICS")  
(xi) SEQUENCE DESCRIPTION:SEQ ID NO:X:  
This sequence is intentionally skipped  
  
Please also adjust the "(iii) NUMBER OF SEQUENCES:" response to include the skipped sequence(s).
- 9        Skipped Sequences (NEW RULES) Sequence(s)        missing. If intentional, please use the following format for each skipped sequence.  
<210> sequence id number  
<400> sequence id number  
000
- 10        Use of n's or Xaa's (NEW RULES) Use of n's and/or Xaa's have been detected in the Sequence Listing.  
Use of <220> to <223> is MANDATORY if n's or Xaa's are present.  
In <220> to <223> section, please explain location of n or Xaa, and which residue n or Xaa represents.
- 11        Use of <213>Organism (NEW RULES) Sequence(s)        are missing this mandatory field or its response.
- 12        Use of <220>Feature (NEW RULES) Sequence(s)        are missing the <220>Feature and associated headings.  
Use of <220> to <223> is MANDATORY if <213>ORGANISM is "Artificial" or "Unknown"  
Please explain source of genetic material in <220> to <223> section.  
(See "Federal Register," 6/01/98, Vol. 63, No. 104, pp. 29631-32) (Sec. 1.823 of new Rules)
- 13        PatentIn ver. 2.0 "bug" Please do not use "Copy to Disk" function of PatentIn version 2.0. This causes a corrupted file, resulting in missing mandatory numeric identifiers and responses (as indicated on raw sequence listing).  
Instead, please use "File Manager" or any other means to copy file to floppy disk.

|||||

PCT09

## RAW SEQUENCE LISTING

PATENT APPLICATION: US/09/763,777

DATE: 03/07/2001

TIME: 15:12:46

Input Set: A:\Seqlist.txt

Output Set: N:\CRF3\03072001\I763777.raw

Does Not Comply  
Corrected Diskette Needed

for 1-2

4 <110> APPLICANT: Thakur, Madhukar L.  
6 <120> TITLE OF INVENTION: Imaging With TC-99M Labeled  
7 Fibrin-Alpha-Chain Peptide  
9 <130> FILE REFERENCE: THA01-NP003  
11 <140> CURRENT APPLICATION NUMBER: US/09/763,777  
12 <141> CURRENT FILING DATE: 2001-02-16  
14 <150> PRIOR APPLICATION NUMBER: 60/096,803  
15 <151> PRIOR FILING DATE: 1998-08-17  
17 <160> NUMBER OF SEQ ID NOS: 5  
19 <170> SOFTWARE: FastSEQ for Windows Version 4.0  
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see item 12 on Enr Summary Sheet

RAW SEQUENCE LISTING  
PATENT APPLICATION: US/09/763,777

DATE: 03/07/2001  
TIME: 13:12:46

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81 <223> OTHER INFORMATION: at amino acid number 6 Xaa designates Aba which is  
82 4-aminobutyric acid  
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85 D-alanine  
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see 1.823 of new Sequence Rules  
for explanation.

These two  
numeric identifiers  
are mandatory  
when n's or Xaa's  
are shown.

VERIFICATION SUMMARY

PATENT APPLICATION: US/09/763,777

DATE: 03/07/2001

TIME: 13:12:47

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L:12 M:271 C: Current Filing Date differs, Replaced Current Filing Date  
L:88 M:258 W: Mandatory Feature missing, <221> not found for SEQ ID#:5  
L:88 M:258 W: Mandatory Feature missing, <222> not found for SEQ ID#:5  
L:88 M:340 W: (4b) "n" or "Xaa" used: Feature required, for SEQ ID#:5

[illegible]

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Clean Copy (With Amendments  
Incorporated As Indicated in Attached  
Preliminary Amendment)

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**IMAGING WITH TC-99M LABELED FIBRIN- $\alpha$ -CHAIN PEPTIDE**

5 **GOVERNMENT RIGHTS IN THE INVENTION**

This invention was made with government support under grant R41 HL 59769-01 (MLT) awarded by the National Institutes of Health. The government has certain rights in the invention.

10

**CROSS REFERENCE TO RELATED APPLICATIONS**

This application claims priority under 35 U.S.C. § 119 based upon U.S. Provisional Patent Application No. 60/096,803 filed August 17, 1998.

15

**FIELD OF THE INVENTION**

20 The present invention generally relates to the field of nuclear medicine and, more particularly, to compositions for radiolabeled agents for imaging mammalian tissue or cells, compositions for radiolabeling agents that bind to mammalian tissue or cells, compositions for radiolabeling agents that bind to fibrin, and methods of using said compositions.

25

**BACKGROUND OF THE INVENTION**

30 Development of radioactive agents for "hot spot" imaging of deep venous thrombosis (DVT) and pulmonary embolism (PE) has been the subject of many investigations for more than two decades. (Thakur ML, Coleman RE, Hoist JH, Welch M. Indium-111 labeled platelets: Studies on preparation and *in vivo* and *in vitro* functions. *Thrombosis Research* 9:345-354, 1976; Knight LC.



Radiopharmaceuticals for thrombus detection. *Seminars in Nucl Med* XX:52-67, 1990; Koblik PD, DeNardo GL, Berger HJ. Current status of Immunoscintigraphy in the detection of thrombosis and thromboembolism. *Seminars in Nucl Med* XIX:221-231, 1989; Thakur ML. Potential of radiolabeled antiplatelet antibodies in the detection of vascular thrombi. In: S.C. Srivastava, ed. *Radiolabeled monoclonal antibodies for imaging and therapy*. Plenum Publishing Co., NATO ASI, series 152, 1988; Thakur ML. Scintigraphic imaging of venous thrombosis: A state of the art. *Thrombotic and Hematologic Disorders* 5:29-36, 1992). One approach to “hot spot” imaging has been to radiolabel platelets, which form a major biochemically active constituent of a thrombus. A large number of agents, therefore, have been evaluated that would target platelets on the assumption that radiolabeled platelets will accrete on an occult thrombus and thereby facilitate its detection by external scintigraphy. Platelets have been labeled *in vitro* using such agents as In-111-oxine which internalizes and binds to platelet cytoplasmic components. (Thakur ML et al., 1976). Platelets have also been labeled *in vivo* using radiolabeled proteins or peptides that are specific for platelet surface glycoprotein complex IIb-IIIa (Knight LC, 1990; Koblik PD et al., 1989; Thakur ML, 1988; Thakur ML, 1992; Knight LC, Radcliffe R, Maurer AH, Rodwell JD, Alvarez VL. Thrombus imaging with Tc-99m synthetic peptides based upon the binding domain of a monoclonal antibody to activated platelets. *J Nucl Med* 35:282-288, 1994; Knight LC, Maurer AH, Romano JE. Comparison of Iodine-123-Disintegrins for Imaging Thrombi and Emboli in a Canine Model. *J Nucl Med* 37:476-482, 1996; Pearson DA, Lister-James J, McBride WJ, Wilson DM, Martel LJ, Civitello ER, Dean RT. Thrombus imaging using Tc-99m labeled high potency GPIIb./IIIa receptor antagonist. Chemistry and initial biological studies. *J Med Chem* 39:1372-1382, 1996; Lister-James J, Vallabhajosula S, Moyer BR, Pearson DA, McBride BJ, De Rosch MA, Bush LR, Machac J, Dean RT. Pre-Clinical Evaluation of Technetium-99m platelet receptor-binding platelet. *J Nucl Med* 38:105-111, 1997; Line BR, Crane P, Lazewatsky J, Barrett JA, Cloutier D, Kagan M, Lukasiewicz R, Holmes RA. Phase I trial of DMP444, a new thrombus imaging agent. *J Nucl Med* 37:117P, 1996; Barrett JA, Crocker AC, Damphousse DJ, Heminway SJ, Liu S, Edwards DS, Lazewatsky JL, Kagan M, Mazaika TJ, Carroll TR. Biological evaluation of 99mTc cyclic glycoprotein IIb/IIIa receptor antagonists in the canine arteriovenous shunt

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and deep vein thrombosis models: Effects of chelators on biological properties of [99mTc]chelator—peptide conjugates. *Bioconjugate Chem* 7:203-208, 1996). Despite the success achieved with these agents in experimental animals and in limited human subjects, only one agent, AcuTect, the Tc-99m labeled peptide P-280, has recently been approved for clinical use. AcuTect is expected to detect acute but not chronic venous thrombosis (AcuTect. Diatide, Inc. *J Nucl Med* 39(10):19N, 1998) or pulmonary embolism, which may harbor activated platelets only sparingly.

A second approach to “hot spot” imaging has been to radiolabel proteins involved in clot formation. During the vessel wall injury, coagulation proteins are activated sequentially and generate the enzyme thrombin. Thrombin cleaves plasma fibrinogen into fibrin monomers, which then polymerize around the platelets and hold them in place as a clot. Fibrin therefore remains an integral part of DVT, fresh or old, and embolized in the lungs or elsewhere in the body. It is primarily for these reasons that I-125-fibrinogen enjoyed popularity for external detection of DVT for a long time (Knight LC, 1990; Koblik PD et al., 1989; Thakur ML, 1992). However, it is no longer available commercially. Iodine-123-fibrinogen and many antifibrin monoclonal antibodies labeled with various radionuclides have also been evaluated. (Koblik PD et al., 1989). However, for many reasons such as the long circulation times or poor image quality, agents other than I-125-fibrinogen did not make it into common nuclear medicine practice.

A third approach to “hot spot” imaging of DVT and PE is to radiolabel antifibrin peptides. The feasibility of this approach has not been previously investigated. One peptide of particular interest is the N-terminus tripeptide, H-Gly-Pro-Arg-OH (SEQ ID NO:1), of fibrin- $\alpha$ -chain, which was reported by Laudano and Doolittle to be an inhibitor of fibrinogen/thrombin clotting. (Laudano AP, Doolittle RF. Synthetic peptide derivatives that bind to fibrinogen and prevent the polymerization of fibrin monomers. *Proc Natl Acad Sci* 75:3085-3089, 1978). The investigators observed that H-Gly-Pro-Arg-Pro-OH (SEQ ID NO:2) analog of the tripeptide was an even more potent inhibitor of fibrinogen/thrombin clot by binding to C-terminus portion of the  $\gamma$ -chain of fibrin and preventing fibrin polymerization. More recently, Kawasaki et al prepared several more analogs and found that a pentapeptide, H-Gly-Pro-Arg-Pro-Pro-OH (SEQ ID NO:3) had the highest fibrinogen/thrombin clotting inhibiting activity. (Kawasaki K, Miyano M, Hirase K,

Iwamoto M. Amino acids and peptides. XVIII. Synthetic peptides related to N-terminus portion of fibrin  $\alpha$ -chain and their inhibitory effect on fibrinogen/thrombin clotting. *Chem Pharm Bull* 41:975-977, 1993).

5           The present invention comprises composition for diagnostic imaging of mammalian cells and tissue. The composition comprises amino acids joined to a linker, which is bound to a moiety that is chelated to a radionuclide. In one of the embodiments, the present invention is a pentapeptide labeled with Tc-99m, that facilitates imaging of DVT and PE.

10

### **DEFINITIONS**

          “TP 850” means the decapeptide, Gly-Pro-Arg-Pro-Pro-Aba-Gly-Gly-(D)-  
15   Ala-Gly. (SEQ ID NO:5).

### **SUMMARY OF THE INVENTION**

20           The present invention comprises a composition for imaging mammalian cells and tissue and method of using said composition.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

**Fig. 1.** The amino acid sequence and the proposed structure of Tc-99m-TP850.

**Fig. 2.** A composite of two HPLC elution spectra obtained under identical  
30   conditions of solvent composition, flow rate, and column. The x axis in both panels is time in minutes and the y axis is radioactivity peak height in  $\mu$ V. The diagonal line is the percent solvent composition. The upper panel is the elution profile of Tc-99m-TP 850 that was injected into the rabbit, and the lower panel is that of the urine sample collected from the rabbit 3 hrs later. Note that the major proportion of the

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radioactivity eluted in the urine has the retention time (Rt) similar to that in the sample injected. The radioactivity at Rt 4 is unbound Tc-99m. The small radioactivity peaks at Rt 6.2 and 9.08 are considered as impurities in the sample.

5 The quantity of the peptide injected was small and was not detectable at 280 nm.

**Fig. 3.** An anterior image of a rabbit obtained at 3 hr post-injection. A small thrombus induced by stimulating electrode in the right arm (arrowhead) and PE in both upper lobes of the lungs (long arrows) are detectable. Also seen in the right side of the neck (short arrows) is radioactivity accumulated in the incision. The  
10 radioactivity in the heart and sinus can be seen.

**Fig. 4.** An anterior image of a rabbit obtained at 1hr 20 min post-injection. A clot induced by stimulating electrode (clot/blood = 6.5) and the one by thrombine-soaked suture (clot/blood = 3.7) are detectable. In addition, radioactivity in the heart, thyroid, and paranasal sinuses can be seen. Free Tc-99m in preparation was  
15 approximately 3%.

**Fig. 5.** Anterior gamma camera images of a rabbit which was injected with 2 mCi Tc-99m-TP850 2 hr and 30 min previously. A clot due to thrombine-soaked suture in the right (arrow) jugular vein and due to stimulating electrode in the left (arrow) are detectable. The activity due to some free Tc-99m in the thyroid can also  
20 be seen. As stated in the text, the electrode clot had 7.1 times more Tc-99m than that in the equal weight of blood and the thrombin clot had 3.6 times more Tc-99m than in the blood. The lower part of the radioactivity is in the heart.

**Fig. 6.** A composite of three images from one rabbit, in which PE was induced 24 hr prior to the i.v. administration of 2.4 mCi of Tc-99m-TP 850. The  
25 scintiphoto in the left panel of the figure was obtained at 1 hr 15 min post-injection in the anterior position. It shows abnormal accumulation of radioactivity in both lungs (arrow). A clot formed spontaneously in the left neck where the incision was made for the placement of the PE introducer sheath is also seen in the scintiphoto given in the left panel of the figure. At the conclusion of in vivo scintigraphy, the  
30 heart and lungs were excised, spread for clarity, and then imaged under a gamma camera, as well as x-rayed. The x-ray image (center panel) shows a tantalum mixed clot in the left lung which corresponds to the shape of a clot seen in the left lung (anterior scintiphoto in the left panel of the figure), as well as to the left lung clot seen in the gamma camera image of the excised lungs and heart given in the right

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panel of the figure. The clot seen in the right lung, in both in vivo (arrow, left panel) and ex vivo (arrow, right panel) images is not seen by x-ray (center panel) because it is free of tantalum. This indicates that this piece of clot may have formed without tantalum in it and lodged in the right lung. Both lung clots were separated, weighed, and counted for radioactivity. The clot in the left lung had three times more and the one in the right lung had 6.1 times more activity than in the unit weight of blood. This clot in the neck had 3.2 times more activity than in the unit weight of blood. Residual blood radioactivity in the heart (H) can also be seen in the right panel of the figure.

### DETAILED DESCRIPTION

#### 15 Materials and Methods

##### i) Preparation of peptide

For this study, a group of four amino acids, Gly-(D)-Ala-Gly-Gly (SEQ ID NO:4) (GAGG) was chosen as a chelating moiety. Through their NH<sub>2</sub> groups these peptides provide an N<sub>4</sub> configuration for a strong chelation of Tc-99m. Rather than the conventional post-synthesis conjugation, the tetrapeptide chelating moiety permitted the modification of the primary peptide at the C terminus during the synthesis. Furthermore, during the synthesis, an additional amino acid, Aba ((D)-4-aminobutyric acid), was inserted as a spacer between the chelating moiety and the primary peptide. The purpose of inserting Aba as a spacer was to minimize any possible steric hindrance resulting from the Tc-99m complex. The synthesis of this modified peptide was one hybrid process which eliminated the multi-step, lengthy, and frequently inefficient conjugation procedure, yet provided a chelating group for a strong chelation of Tc-99m. The resultant decapeptide, Gly-Pro-Arg-Pro-Pro-Aba-Gly-Gly-(D)-Ala-Gly (SEQ ID NO:5) which has an expected M.W. of 850, is hereafter referred to as TP 850.

The peptide was custom synthesized (PeptidoGenic Research Co., Inc. Livermore, CA) using a Shimadzu solid phase synthesizer (Shimadzu, Columbia, MD) and separated using HFIsil, C-18, 5 micron preparative HPLC column. Ion

spray mass analysis was performed using Perkin Elmer's Sciex APZ I mass spectrometer (Norwalk, CT). Using this chelating moiety and facility several peptides have previously been prepared and labeled with Tc-99m in our laboratory.

- 5 (Thakur ML, Pallela VR, Consigny PM. Tc-99m-TP 1201 for imaging thromboembolism. *Radiology* 205:267P, 1997; Pallela VR, Consigny PM, Shi R, Thakur ML. Imaging vascular thrombosis with Tc-99m-TP 1300 peptide derived from active domain of thrombospondin. *J Nucl Med* 39:64P, 1998; Pallela VR, Consigny PM, Shi R, Thakur ML. Tc-99m-labeled Fibrin- $\alpha$ -chain peptide analog  
10 for imaging vascular thrombosis. *Eur J Nucl Med* 25:878, 1998; Pallela VR, Thakur ML, Consigny PM, Rao PS, Vassileva-Belnikolovska D, Shi R. Imaging thromboembolism with Tc-99m labeled thrombospondin receptor analogs TP-1201 and TP-1300. *Thrombosis Research* 93:191-202, 1999; Pallela VR, Thakur ML, Chakder S, and Rattan S. 99mTc-labeled vasoactive intestinal peptide receptor  
15 agonist: Functional studies. *J Nucl Med* 40:352-360, 1999).

ii) Radiolabeling and quality control:

- 20 Fifty  $\mu$ g of TP 850 was dissolved in 10  $\mu$ l 10% acetonitrile in water, then 200  $\mu$ l of 0.1 M  $\text{Na}_3\text{PO}_4$  were added, followed by 10-30 mCi Tc-99m in 200  $\mu$ l isotonic saline previously reduced with 100  $\mu$ g  $\text{SnCl}_2$  in 10  $\mu$ l of 0.05 M HCl. Lately, with a new batch of high purity  $\text{SnCl}_2$  (Sigma Chemicals, St. Louis, MO) we have been able to reduce the  $\text{SnCl}_2$  to 10  $\mu$ g. The reaction mixture was then  
25 incubated for 30 min in a boiling water bath. The product was examined by HPLC (Rainin, Emeryville, CA) using a reverse phase C-18 column and gradient solvents of 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). The gradient was such that at zero minutes solvent A was 90%, and at 30 min solvent B was 100%. The flow rate was 1 ml/min. The HPLC was equipped with a u.v. detector set at 278 nm,  
30 a 2" NaI (TI) gamma counter, and a rate meter.

iii) Stability of Tc-99m-TP 850:

The stability of the radiolabeled peptide at 22° C was examined by HPLC for up to 24 hrs as determined by the characteristic retention time of the radioactivity peak. The in vivo stability was examined by injecting approximately 2 mCi Tc-99m-TP 850 preparation, collecting urine 3 hrs later, and analyzing a 20 µl portion of the urine by HPLC.

iv) Fibrin binding:

The ability of Tc-99m-TP 850 to bind to rabbit, dog, and human fibrin was examined in vitro. Institutional approval was obtained to draw human blood and to perform all animal experiments. Approximately 10 ml of venous blood was obtained from a healthy human volunteer and from a normal young adult dog and a rabbit. No anticoagulating agent was added to the blood. After the blood was clotted, from each blood sample, one ml serum samples were dispensed in four separate test tubes and approximately 25 µCi of Tc-99m-TP 850 (specific activity approximately 340 Ci/m mol) were added to each tube and the reagents were gently mixed. Thrombin (six i.u.) was then added to the first two test tubes and an equal volume of saline to the other two. The contents were gently mixed and allowed to incubate for 10 min at 37°C. The test tubes were then centrifuged (2000 g x 10 min), the supernatant carefully removed, and the fibrin clots in the first two test tubes were washed twice with 2 ml 0.9% NaCl. Following centrifugation, the washing liquid was combined with the supernatant. Radioactivity associated with the clot and the supernatant were measured and calculated as the percent of total activity found in the compact fibrin clot.

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v) Inhibition of platelet aggregation:

Seventeen ml of venous blood from a rabbit and a dog were collected in 3 ml Acid Citrate Dextrose A (ACD A), centrifuged at 180 g for 10 min and platelet rich plasma (PRP) was separated. (Thakur ML, Coleman RE, Hoist JH, Welch M. Indium-111 labeled platelets: Studies on preparation and in vivo and in vitro functions. *Thrombosis Research* 9:345-354, 1976). Aggregation studies were performed using a Chronolog (Havertown, PA) aggregometer. For each study, increasing quantities of TP 850 and 4  $\mu$ M ADP were added to 500  $\mu$ l PRP containing approximately  $1.5 \times 10^8$  platelets, stirring at 37°C. Aggregation in the absence of TP 850 was considered 100% and IC<sub>50</sub> values were determined using the quantity of TP 850 that inhibited aggregation by 50%.

vi) Blood clearance:

All animal protocols were approved by the Institutional Animal Care and Use Committee and were strictly followed. Blood clearance of the agent was examined in adult New Zealand white rabbits weighing between 3 to 3.5 kg. Each rabbit was anesthetized by an i.m. injection of ketamine (30 mg/kg) and zylaxine (5 mg/kg). Thereafter a 23 gauge catheter was inserted in the right ear artery and connected to a leuer lock (Burron Med. Inc., Bethlehem, PA). The patency of the catheter was maintained by the administration of 6 i.u. heparin per ml of sterile 0.9% NaCl administered through the leuer lock. This catheter was used for drawing 0.5 ml blood samples in duplicate at 1,5,10,15, and 30 min, and then at 1, 2, and 3 hrs after radionuclide injection. Before each sample collection enough blood was withdrawn to replace saline, which avoided the dilution of each blood sample collected.

The marginal vein of the contralateral ear was used for injecting radioactive agents. The radioactivity in the syringe was measured before and after injection to determine the dose injected, and a suitable Tc-99m standard solution was prepared. Blood samples were then weighed, radioactivity counted, and results were expressed



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as percent injected dose per gram (% I.D./g) of blood and plotted as a function of time.

5

vii) Tissue distribution studies:

Tissue samples were harvested from three rabbits three hrs after the administration of Tc-99m-TP 850. Tissues were weighed, radioactivity associated with each tissue, and a reference standard solution of Tc-99m prepared at the time of injection was determined. Radioactivity was expressed as % injected dose/g (% I.D./g) of tissue. Results were averaged and standard deviation was determined.

15

viii) Inducing DVT:

Each of the eight adult (male or female) New Zealand white rabbits, weighing between 3-3.5 kg was anesthetized as described above, the right cubital vein and/or jugular vein was exposed, and a stimulating electrode was inserted (Leadley RJ, Humphrey WR, Erickson LA, Shebuski RJ. Inhibition of thrombus formation by Endothelin-1 in canine models of arterial thrombosis. *Thrombosis and Haemostasis* 74:1583-1590, 1995). The electrode was constructed from a 26-gauge stainless steel hypodermic needle bent at a 90° angle and attached to a 30-gauge, Teflon insulated silver coated copper wire. The needle was inserted into the vessel and then gently pulled so that it was in contact with the endothelial lining of the vessel and secured in place with a flared sleeve inserted over the copper wire. The second electrode was applied to the tongue of the rabbit. The stimulating electrode was attached to the anode and the other electrode to the cathode of a power supply. A 450 µA current was then applied and 10 min later 2 mCi of Tc-99m TP 850 (specific activity approximately 510 Ci/m mol) in 2 ml 0.9% solution was injected through a marginal ear vein. Radioactivity in each dose was measured before and after administration and recorded. A suitable reference solution with a known quantity of Tc-99m was also prepared. In two additional rabbits thrombus was induced by inserting a

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thrombin-soaked suture into a jugular vein 10 min prior to the administration of Tc-99m-TP 850. Serial gamma camera images of the rabbit, in the supine position, were then obtained for up to four hours, using a GE Starcam gamma camera (GE, Milwaukee, WI) coupled to a low energy parallel hole collimator. For each image a total of 350,000 counts were collected.

ix) Inducing pulmonary embolism:

Pulmonary emboli were induced in six additional rabbits. Radio opaque pulmonary emboli were prepared by drawing 0.5 to .75 ml blood, through a 23G butterfly needle inserted in the marginal ear vein, into a one ml syringe containing 15 mg tantalum powder and 6 i.u. of thrombin. The contents of the syringe were then mixed gently and a clot was allowed to form and harden for 20 min. The clot was removed from the syringe and a one cm long piece of the clot was drawn in an introducer sheath (6Fr, Pinnacle, MediTech, Watertown, MA), which was then inserted into a previously isolated jugular vein and advanced into the right atrium. The clot was then flushed from the sheath with isotonic saline. The position of the tantalum containing clots was confirmed by recording a chest x-ray of the animals before the administration of Tc-99m-TP 850 and an x-ray of the excised lungs after sacrifice. Following clot administration and the confirmation of its localization by x-ray, Tc-99m-TP 850 was injected and the rabbits were imaged as described in the previous section. Four animals with PE were allowed to recover from the surgery. Two rabbits were injected with Tc-99m-TP 850 24 hrs later and the other two were injected 48 hours later.

Upon the conclusion of imaging for PE or DVT, each rabbit was given an intravenous injection of heparin (1000 i.u.) and was then euthanized with sodium pentobarbital (100 mg/kg). A blood sample was drawn, and then the lungs and heart were excised, radiographed, and the clots were harvested. The clots and blood were weighed, radioactivity associated with them was counted, and clot/blood ratios were determined.

Resultsi) Peptide radiolabeling, quality control, and stability:

5 The purity of the peptide as determined by HPLC analysis was > 90%. The expected M.W. of the peptide was 850, and the one observed by mass spectroscopic analysis was 849.4. The proposed structure of Tc-99m labeled TP 850 is given in **Fig. 1**, which shows that Tc-99m is bound to the chelating moiety with N<sub>4</sub> configuration. The Tc-99m labeling consistently produced > 95% yield. HPLC analysis indicated that > 90% of that activity was eluted in a single peak at retention time (Rt) of seven min. A small quantity (< 5%) of radioactivity was eluted at a Rt of 6.2 min and any unbound Tc-99m at a Rt of 3.5 min. An elution profile is given in **Fig. 2**.

15 The preparations of Tc-99m-TP 850 were stable at 22°C for 24 hrs. HPLC analysis of a urine sample (**Fig. 2**) collected at three hrs after an injection of Tc-99m-TP 850 showed that the radioactivity elution profile was similar to that of the preparation injected, and that the retention time of the radioactivity peak in the urine sample was similar to that of the radioactivity sample injected. This suggested that  
20 the small peptide was not susceptible to a rapid in vivo proteolysis.

ii) Blood clearance and tissue distribution:

The blood clearance was biphasic with a  $t_{1/2-\alpha}$  being approximately four min  
25 (20%) and  $t_{1/2-\beta}$  being approximately 13 min (80%). Examination of three hr tissue distribution of Tc-99m-TP 850 indicated that the highest radioactivity was in the kidneys ( $0.10 \pm 0.086$  % I.D./g), suggesting that the kidneys were the primary route of excretion. The liver uptake was  $0.016 \pm 0.014$  % I.D./g and intestine  $0.01 \pm 0.009$  % I.D./g. The blood uptake at this time was only  $0.007 \pm 0.004$  % I.D./g. This  
30 small proportion of radioactivity in circulating blood facilitated the imaging of vascular thrombi. Radioactivity in all other tissues was unremarkable.

ii) Fibrin binding and inhibition of platelet aggregation:

TP 850 radioactivity associated with human, dog, and rabbit fibrin was  $42 \pm 2\%$ ,  $60 \pm 39\%$ , and  $56 \pm 2.5\%$ , respectively. The  $IC_{50}$  values for the dog and rabbit platelet aggregation inhibition were  $236 \mu\text{m}$  and  $167 \mu\text{m}$ , respectively. These data justified the use of rabbit as a model for studies with Tc-99m-TP 850.

10 iii) Imaging DVT and PE:

Although Tc-99m-TP 850 cleared rapidly from the blood, cardiac blood pool activity was detectable in all animals at all imaging times. Radioactivity in the sinus was also detectable in all animals studied. This was consistent with the results of Tc-99m-TP1201 and Tc-99m-TP1300, the activated platelet receptor specific thrombospondin analogs studied previously in our laboratory. (Pallela VR, Thakur ML, Consigny PM, Rao PS, Vassileva-Belnikolovska D, Shi R. Imaging thromboembolism with Tc-99m labeled thrombospondin receptor analogs TP-1201 and TP-1300. *Thrombosis Research* 93:191-202, 1999). Unlike these two agents, however, no Tc-99m-TP 850 radioactivity was seen either in the bone or in the bone cartilage. All fresh DVT and PE were detectable by Tc-99m-TP 850 generally within 90-120 min post-injection. Clots that formed spontaneously in surgical incision or ligated vessels were also detectable. Similarly, PE that were formed by a piece of a clot broken off or separated from a clot that was injected into the right atrium were imageable. An example is given in **Fig. 3**, in which an electrode-induced clot in the right forearm, two PE, one in each upper lobe of the lungs, and radioactivity accumulated in the incision was seen. In this animal, the forearm clot to blood radioactivity ratio was 12, and the PE to blood 1.3 (L), and 2.1 (R). The radioactivity associated with the clots was 0.087% I.D./g, 0.006 % I.D./g and 0.007% I.D./g, respectively.

The clot/blood ratios in the rabbits studied ranged from 1.2 to 12. Many times these clots were small and could not be easily separated without the vessel wall or adjoining fatty tissue. Similarly, tantalum was embedded in many PE.

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Consequently, the weight contributed by the additional tissue or tantalum, resulted in the low and variable radioactivity per unit weight of clots, PE, or DVT.

Fig. 4 shows an anterior image of a rabbit given Tc-99m-TP 850 one hr and 20 min previously. A clot in the right jugular vein induced by stimulating electrode and the one in the left jugular vein induced by thrombin-soaked suture were detectable. The clot to blood ratios were 6.5 and 3.7, respectively. The clot radioactivity was 0.035% I.D./g and 0.02% I.D./g. In this animal, the radioactivity was also seen in the thyroid due to 3.5% unbound free Tc-99m that was injected.

Fig. 5 shows an anterior image of a rabbit obtained at 150 min after the injection of Tc-99m-TP 850 into which thrombin-soaked suture was placed in the right jugular vein and a stimulating electrode clot was formed in the left jugular vein. Both clots were detectable with the electrode clot to blood ratio of 7.1 and the suture soaked thrombin clot/blood ratio of 3.6. Included in each suture clot was the weight of the suture itself which artificially decreased the clot/blood radioactivity ratios. The radioactivity incorporated into these clots measured 0.046% I.D./g and 0.024% I.D./g of the weight of the clot.

Fig. 6 is an anterior image of a rabbit with PE in both lungs induced 24 hrs previously. The image was positive at one hr and 15 min post-injection of Tc-99m-TP 850. Lungs were excised, imaged, and x-rayed. The location of the clots was corroborated. The clots were then retrieved, weighed, and associated radioactivity was measured. The clot to blood ratios were 6.1 for the right clot and 3.0 for the one in the left clot. The radioactivity in the clot was 0.021% I.D./ and 0.01% I.D./g.

In contrast, 48 hr old clots were neither detectable by scintigraphy nor by x-ray. This suggested that they were lysed and had disappeared. This is consistent with the high fibrinolytic activity in rabbits. (Didisheim P. Animal models useful in the study of thrombosis and antithrombotic agents. *Prog in Hemostasis and Thrombosis* Spaet TH, ed. Grune and Stratton: New York, pp. 165-197, 1976; Doolittle RF, Omcley JL, and Surgenor DM. Species differences in the interaction of thrombin and fibrinogen. *J Biol Chem* 237:3123, 1962; Gallimore MJ, Nulkar MV, and Shaw JTB. A comparative study of the inhibitors of fibrinolysis in human, dog, and rabbit blood. *Thromb Diath Haemorrh* 14:145-158, 1965; Hawkey CM, Fibrinolysis in animals. In *The Haemostatic Mechanism in Man and Other Animals*, MacFarlane RG, ed. Academic Press: London, pp. 143-150, 1979; Mason

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RG and Read MS. Some species differences in fibrinolysis blood coagulation. *J Biomed Mater Res* 5:121-128, 1971; Craig IH, Bell FP, and Schwartz CJ. Thrombosis and atherosclerosis: The organization of pulmonary thromboemboli in the pig. *Exper Mol Path* 18:290-301, 1973). The influence of any anticoagulant therapeutic intervention has not yet been studied.

### Discussion

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In the USA, more than 378,000 patients are hospitalized annually for DVT, and more than 103,000 for PE. (Vital and Health Statistics. Series 13: Data from National Health Survey Ditts Publication No. (PHS)95-1783, 1993). These conditions, despite modern techniques, contribute to more than 200,000 deaths every year. The clinical diagnosis of DVT and PE is unreliable (Burke B, Sostman D, Carroll B, and Witty LA. The diagnostic approach to deep venous thrombosis. *Clinics in Chest Medicine* 16:253-1568, 1995; Worsley DF, Alavi A, Palevsky, HI. Role of radionuclide imaging in patients with suspected pulmonary embolism. *Radiologic Clinics of North America* 31:849-859, 1993), and PE is an often underestimated, underdiagnosed, and undertreated disease. (Janata-Schwatzek K, Weiss K, Riezinger I, Bankier A, Domanovits H, Seidler, D. Pulmonary Embolism: Diagnosis and treatment. *Seminars in Thrombosis and Hemostasis* 22:33-52, 1996).

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Venography is invasive and other modalities have limitations. Spiral CT, MRI, and ventilation-perfusion (VQ) scans remain the leading diagnostic tools for its diagnosis. In spiral CT, a number of interpretive pitfalls exist in assessing images of PE and MRI is not likely to replace CT. Although CT has better resolution and less sensitivity to moving lung artifacts, its pitfalls, and use of frequently allergic contrast agents have led investigators to rely upon VQ scanning. VQ scanning itself is a cold spot imaging techniques and can only predict low or high probability of PE. For many clinicians this type of diagnosis is inadequate.

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In principle, external scintigraphic techniques aided by the use of a suitable radiopharmaceutical can provide hot spot images and can

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fulfill the need since scintigraphic techniques are non-invasive, and can scan the entire body of a patient without unreasonable inconvenience or added morbidity to the patient.

5 During the past few years, a large number of radiopharmaceuticals have been investigated as potential agents to localize DVT or PE. Since thrombi are largely composed of fibrin, platelets and other cells entrapped in the fibrin network, much attention was drawn to the use of radioiodine labeled fibrinogen and In-111 labeled platelets.

10 In many ways, radiolabeled platelets should be a simple and ideal agent, for they form a major and the most biologically active constituent of a thrombus. However, radiolabeled platelets have been less attractive largely due to their long life span (8 days) that elevated background radioactivity for several days after their administration. The slow clearance of radioactivity causes delay in diagnosis due to  
15 suboptimal lesion to background radioactivity ratios. The platelets must also be labeled in vitro which requires skilled personnel. Furthermore, in the presence of anticoagulant therapy, heparin in particular, when accretion of fresh platelets is impeded, In-111 platelet scintigraphy is less successful. An array of antibodies, the majority of them specific for IIb and IIIa glycoprotein complex on the platelet  
20 surface, have also been investigated. Success with these has been limited for a variety of reasons including the lack of specificity, unfavorable pharmacokinetics or cumbersome preparation of the agent. The pros and cons of these and other agents have been described by Knight, Thakur, and Koblik et al.

Prompted by the advancements in science and technology of molecular  
25 biology, recent development of radioactive agents for non-invasive diagnosis of thromboembolism are centered around the use of Tc-99m labeled peptides specific for resting or activated platelets. Peptides are smaller in size and easier to produce than monoclonal antibodies. They are expected to clear more rapidly from circulation than radiolabeled proteins, less likely to induce any immunological  
30 reaction, yet in most cases they enjoy as high a receptor specificity and binding constants as the monoclonal antibodies. Because of the physical characteristic of Tc-99m, the Tc-99m labeled peptides have become even more attractive biomolecules for diagnostic imaging than antibodies labeled with In-111. Technetium-99m is easy to obtain worldwide, inexpensive, and decays with gamma

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ray energy (140 KeV, 90%) that can be efficiently detected by gamma cameras, planar or tomographic. It has a half-life (6 hr) that is long enough to perform examinations before excessive radioactive decay has occurred, yet not too long to persist in the body long after the examinations have been carried out and to give  
5 excessive radiation dose to the subject.

All of the peptides evaluated thus far are specific for platelet glycoprotein receptor complex IIb IIIa. Among them, one peptide, Tc-99m-P280 was recently approved by the FDA under the trade name AcuTect. As per the manufacturer's description, the peptide is expected to detect only acute thrombi but not old clots or  
10 PE. A primary reason for this is physiologic, in that fresh platelets, to which AcuTect may bind in vivo, seldom accumulate in chronic clots or PE. A different approach to the problem is therefore necessary that will permit imaging of DVT as well as PE.

The coagulation process described earlier generates fibrin monomers that  
15 form a substantial part of a clot. The actual quantity of fibrin content may vary from clot to clot, but generally it is expected to be the same as the fibrinogen of the blood which in most adults is as high as five grams per 100 grams of plasma proteins. Since fibrin exists on both the surface and within clots that are forming or dissolving, the development of Tc-99m labeled peptide, specific for fibrin is  
20 appealing. Such agents, in principle, can target fibrin at any stage or state of a clot and reliably image it. For this purpose, one peptide of particular interest is the N-terminus fibrin  $\alpha$ -chain peptide, H-Gly-Pro-Arg-OH (SEQ ID NO:1), which was reported by Laudano and Doolittle to be an inhibitor of fibrinogen/ thrombin clotting (13). The  $\alpha$ -chain of fibrin begins with the same tripeptide sequence in many  
25 animal species as well as in humans. These investigators observed that H-Gly-Pro-Arg-Pro-OH (SEQ ID NO:2) analog of the tripeptide was an even more potent inhibitor of fibrinogen/thrombin clotting than the tripeptide itself since it also bound to C-terminus portion of the  $\gamma$ -chain of fibrin and prevented fibrin polymerization. More recently, Kawasaki, et al, prepared several more analogs and found that a  
30 pentapeptide, H -Gly-Pro-Arg-Pro-Pro-OH (SEQ ID NO:3) had the highest fibrinogen/thrombin clot inhibiting activity.



**I CLAIM:**

1. A composition having formula I or II:



wherein:

$X_1$  is from zero to twenty natural or synthetic amino acids;

P is a peptide comprising Gly Pro Arg (SEQ ID NO: 1), or an analog or fragment thereof;

$X_2$  is from zero to twenty natural or synthetic amino acids;

Z is a linker comprising one or more natural or synthetic amino acids; and

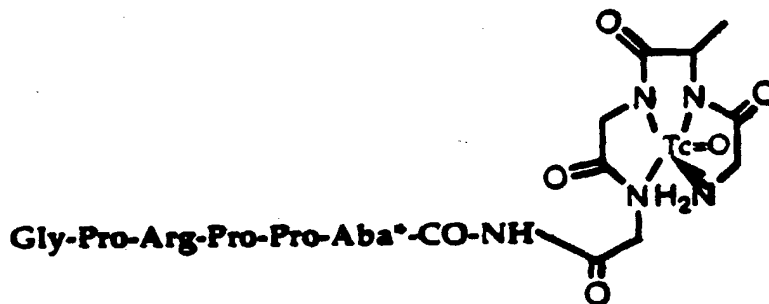
M is a radiolabeling moiety comprised of a chelating moiety capable of complexing with a selected radionuclide.

2. The composition according to Claim 1 comprising SEQ ID NO: 1.

3. The composition according to Claim 1, wherein the radiolabeling moiety is complexed to the radionuclide.

4. The composition according to Claim 3, wherein the radionuclide is technetium-99m.

5. The composition according to Claim 3 having the formula:



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6. The composition according to Claim 1, wherein M comprises Gly -(D)-Ala-Gly-Gly (SEQ ID NO: 4) as a chelating moiety for a radionuclide.

5 7. A method of imaging mammalian cells or tissue, comprising administering a diagnostically effective amount of the composition of Claim 1 to a mammal at a target site and detecting the composition at said target site.

10 8. The method of Claim 6, wherein said target site is a mammalian thrombus.

9. A method of imaging thrombus in a mammal, comprising:  
administering a diagnostically effective amount of a composition that binds  
to fibrin, said composition having a radiolabeling moiety; and  
detecting said composition at a site of said thrombus.

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**ABSTRACT**

5           The present invention involves compositions for radiolabeled agents for  
imaging mammalian tissue or cells, compositions for radiolabeling agents that bind  
to mammalian tissue or cells, compositions for radiolabeling agents that bind to  
fibrin, and methods of using said compositions.

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Version with markings to show changes made"

## IMAGING WITH TC-99M LABELED FIBRIN- $\alpha$ -CHAIN PEPTIDE

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### GOVERNMENT RIGHTS IN THE INVENTION

This invention was made with government support under grant R41 HL 59769-01 (MLT) awarded by the National Institutes of Health. The government has  
10 certain rights in the invention.

### CROSS REFERENCE TO RELATED APPLICATIONS

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This application claims priority under 35 U.S.C. § 119 based upon U.S. Provisional Patent Application No. 60/096,803 filed August 17, 1998.

### FIELD OF THE INVENTION

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The present invention generally relates to the field of nuclear medicine and, more particularly, to compositions for radiolabeled agents for imaging mammalian tissue or cells, compositions for radiolabeling agents that bind to mammalian tissue or cells, compositions for radiolabeling agents that bind to fibrin, and methods of using said compositions.

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### BACKGROUND OF THE INVENTION

Development of radioactive agents for "hot spot" imaging of deep venous  
30 thrombosis (DVT) and pulmonary embolism (PE) has been the subject of many investigations for more than two decades. (Thakur ML, Coleman RE, Hoist JH, Welch M. Indium-111 labeled platelets: Studies on preparation and *in vivo* and *in vitro* functions. *Thrombosis Research* 9:345-354, 1976; Knight LC.

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Radiopharmaceuticals for thrombus detection. *Seminars in Nucl Med* XX:52-67, 1990; Koblik PD, DeNardo GL, Berger HJ. Current status of Immunoscintigraphy in the detection of thrombosis and thromboembolism. *Seminars in Nucl Med* XIX:221-231, 1989; Thakur ML. Potential of radiolabeled antiplatelet antibodies in the detection of vascular thrombi. In: S.C. Srivastava, ed. *Radiolabeled monoclonal antibodies for imaging and therapy*. Plenum Publishing Co., NATO ASI, series 152, 1988; Thakur ML. Scintigraphic imaging of venous thrombosis: A state of the art. *Thrombotic and Hematologic Disorders* 5:29-36, 1992). One approach to "hot spot" imaging has been to radiolabel platelets, which form a major biochemically active constituent of a thrombus. A large number of agents, therefore, have been evaluated that would target platelets on the assumption that radiolabeled platelets will accrete on an occult thrombus and thereby facilitate its detection by external scintigraphy. Platelets have been labeled *in vitro* using such agents as In-111-oxine which internalizes and binds to platelet cytoplasmic components. (Thakur ML et al., 1976). Platelets have also been labeled *in vivo* using radiolabeled proteins or peptides that are specific for platelet surface glycoprotein complex IIb-IIIa (Knight LC, 1990; Koblik PD et al., 1989; Thakur ML, 1988; Thakur ML, 1992; Knight LC, Radcliffe R, Maurer AH, Rodwell JD, Alvarez VL. Thrombus imaging with Tc-99m synthetic peptides based upon the binding domain of a monoclonal antibody to activated platelets. *J Nucl Med* 35:282-288, 1994; Knight LC, Maurer AH, Romano JE. Comparison of Iodine-123-Disintegrins for Imaging Thrombi and Emboli in a Canine Model. *J Nucl Med* 37:476-482, 1996; Pearson DA, Lister-James J, McBride WJ, Wilson DM, Martel LJ, Civitello ER, Dean RT. Thrombus imaging using Tc-99m labeled high potency GPIIb./IIIa receptor antagonist. Chemistry and initial biological studies. *J Med Chem* 39:1372-1382, 1996; Lister-James J, Vallabhajosula S, Moyer BR, Pearson DA, McBride BJ, De Rosch MA, Bush LR, Machac J, Dean RT. Pre-Clinical Evaluation of Technetium-99m platelet receptor-binding platelet. *J Nucl Med* 38:105-111, 1997; Line BR, Crane P, Lazewatsky J, Barrett JA, Cloutier D, Kagan M, Lukasiewicz R, Holmes RA. Phase I trial of DMP444, a new thrombus imaging agent. *J Nucl Med* 37:117P, 1996; Barrett JA, Crocker AC, Damphousse DJ, Heminway SJ, Liu S, Edwards DS, Lazewatsky JL, Kagan M, Mazaika TJ, Carroll TR. Biological evaluation of 99mTc cyclic glycoprotein IIb/IIIa receptor

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antagonists in the canine arteriovenous shunt and deep vein thrombosis models:

Effects of chelators on biological properties of [99mTc]chelator—peptide conjugates. *Bioconjugate Chem* 7:203-208, 1996). Despite the success achieved with these agents in experimental animals and in limited human subjects, only one agent, AcuTect, the Tc-99m labeled peptide P-280, has recently been approved for clinical use. AcuTect is expected to detect acute but not chronic venous thrombosis (AcuTect. Diatide, Inc. *J Nucl Med* 39(10):19N, 1998) or pulmonary embolism, which may harbor activated platelets only sparingly.

A second approach to "hot spot" imaging has been to radiolabel proteins involved in clot formation. During the vessel wall injury, coagulation proteins are activated sequentially and generate the enzyme thrombin. Thrombin cleaves plasma fibrinogen into fibrin monomers, which then polymerize around the platelets and hold them in place as a clot. Fibrin therefore remains an integral part of DVT, fresh or old, and embolized in the lungs or elsewhere in the body. It is primarily for these reasons that I-125-fibrinogen enjoyed popularity for external detection of DVT for a long time (Knight LC, 1990; Koblik PD et al., 1989; Thakur ML, 1992). However, it is no longer available commercially. Iodine-123-fibrinogen and many antifibrin monoclonal antibodies labeled with various radionuclides have also been evaluated. (Koblik PD et al., 1989). However, for many reasons such as the long circulation times or poor image quality, agents other than I-125-fibrinogen did not make it into common nuclear medicine practice.

A third approach to "hot spot" imaging of DVT and PE is to radiolabel antifibrin peptides. The feasibility of this approach has not been previously investigated. One peptide of particular interest is the N-terminus tripeptide, <sup>64</sup>[H-Gly-Pro-Arg-OH] (SEQ ID NO:1) of fibrin- $\alpha$ -chain, which was reported by Laudano and Doolittle to be an inhibitor of fibrinogen/thrombin clotting. (Laudano AP, Doolittle RF. Synthetic peptide derivatives that bind to fibrinogen and prevent the polymerization of fibrin monomers. *Proc Natl Acad Sci* 75:3085-3089, 1978). The investigators observed that <sup>1</sup>[H-Gly-Pro-Arg-Pro-OH] (SEQ ID NO:2) analog of the tripeptide was an even more potent inhibitor of fibrinogen/thrombin clot by binding to C-terminus portion of the  $\gamma$ -chain of fibrin and preventing fibrin polymerization. More recently, Kawasaki et al prepared several more analogs and found that a pentapeptide, <sup>1</sup>[H-Gly-Pro-Arg-Pro-~~Pro~~] (SEQ ID NO:3)

Pro-OH (SEQ ID NO:3)  
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(Pro-OH) had the highest fibrinogen/thrombin clotting inhibiting activity. (Kawasaki K, Miyano M, Hirase K, Iwamoto M. Amino acids and peptides. XVIII. Synthetic peptides related to N-terminus portion of fibrin  $\alpha$ -chain and their inhibitory effect on fibrinogen/thrombin clotting. *Chem Pharm Bull* 41:975-977, 1993).

5 The present invention comprises composition for diagnostic imaging of mammalian cells and tissue. The composition comprises amino acids joined to a linker, which is bound to a moiety that is chelated to a radionuclide. In one of the embodiments, the present invention is a pentapeptide labeled with Tc-99m, that facilitates imaging of DVT and PE.

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## DEFINITIONS

15 Ala-Gly (SEQ ID NO:1) "TP 850" means the decapeptide,  $\text{Gly-Pro-Arg-Pro-Pro-Ala-Gly-Gly-(D)-Gly-Pro-Arg-Pro-Pro-Ala-Gly-Gly-(D)-}$

## SUMMARY OF THE INVENTION

20 The present invention comprises a composition for imaging mammalian cells and tissue and method of using said composition.

## 25 BRIEF DESCRIPTION OF THE DRAWINGS

**Fig. 1.** The amino acid sequence and the proposed structure of Tc-99m-TP850.

30 **Fig. 2.** A composite of two HPLC elution spectra obtained under identical conditions of solvent composition, flow rate, and column. The x axis in both panels is time in minutes and the y axis is radioactivity peak height in  $\mu\text{V}$ . The diagonal line is the percent solvent composition. The upper panel is the elution profile of Tc-99m-TP 850 that was injected into the rabbit, and the lower panel is

that of the urine sample collected from the rabbit 3 hrs later. Note that the major proportion of the radioactivity eluted in the urine has the retention time (Rt) similar to that in the sample injected. The radioactivity at Rt 4 is unbound Tc-99m. The small radioactivity peaks at Rt 6.2 and 9.08 are considered as 5 impurities in the sample. The quantity of the peptide injected was small and was not detectable at 280 nm.

**Fig. 3.** An anterior image of a rabbit obtained at 3 hr post-injection. A small thrombus induced by stimulating electrode in the right arm (arrowhead) and PE in both upper lobes of the lungs (long arrows) are detectable. Also seen in the right side of the neck (short arrows) is radioactivity accumulated in the incision. The radioactivity in the heart and sinus can be seen.

**Fig. 4.** An anterior image of a rabbit obtained at 1 hr 20 min post-injection. A clot induced by stimulating electrode (clot/blood = 6.5) and the one by thrombine-soaked suture (clot/blood = 3.7) are detectable. In addition, radioactivity in the heart, thyroid, and paranasal sinuses can be seen. Free Tc-99m in preparation was approximately 3%.

**Fig. 5.** Anterior gamma camera images of a rabbit which was injected with 2 mCi Tc-99m-TP850 2 hr and 30 min previously. A clot due to thrombin-soaked suture in the right (arrow) jugular vein and due to stimulating electrode in the left (arrow) are detectable. The activity due to some free Tc-99m in the thyroid can also be seen. As stated in the text, the electrode clot had 7.1 times more Tc-99m than that in the equal weight of blood and the thrombin clot had 3.6 times more Tc-99m than in the blood. The lower part of the radioactivity is in the heart.

**Fig. 6.** A composite of three images from one rabbit, in which PE was induced 24 hr prior to the i.v. administration of 2.4 mCi of Tc-99m-TP 850. The scintiphoto in the left panel of the figure was obtained at 1 hr 15 min post-injection in the anterior position. It shows abnormal accumulation of radioactivity in both lungs (arrow). A clot formed spontaneously in the left neck where the incision was made for the placement of the PE introducer sheath is also seen in the scintiphoto given in the left panel of the figure. At the conclusion of in vivo scintigraphy, the heart and lungs were excised, spread for clarity, and then imaged under a gamma camera, as well as x-rayed. The x-ray image (center panel) shows a tantalum



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mixed clot in the left lung which corresponds to the shape of a clot seen in the left lung (anterior scintiphoto in the left panel of the figure), as well as to the left lung clot seen in the gamma camera image of the excised lungs and heart given in the right panel of the figure. The clot seen in the right lung, in both in vivo (arrow, 5 left panel) and ex vivo (arrow, right panel) images is not seen by x-ray (center panel) because it is free of tantalum. This indicates that this piece of clot may have formed without tantalum in it and lodged in the right lung. Both lung clots were separated, weighed, and counted for radioactivity. The clot in the left lung had three times more and the one in the right lung had 6.1 times more activity than 10 in the unit weight of blood. This clot in the neck had 3.2 times more activity than in the unit weight of blood. Residual blood radioactivity in the heart (H) can also be seen in the right panel of the figure.

### DETAILED DESCRIPTION

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#### Materials and Methods

##### i) Preparation of peptide

For this study, a group of four amino acids, <sup>Gly (D)-Ala-Gly-Gly (SEQ ID NO.4)</sup> [Gly-(D)-Ala-Gly-Gly] 20 (GAGG) was chosen as a chelating moiety. Through their NH<sub>2</sub> groups these peptides provide an N<sub>4</sub> configuration for a strong chelation of Tc-99m. Rather than the conventional post-synthesis conjugation, the tetrapeptide chelating moiety permitted the modification of the primary peptide at the C terminus during the synthesis. Furthermore, during the synthesis, an additional amino acid, <sup>(D)-4-</sup> <sup>aminobutyric acid</sup> (Aba), was inserted as a spacer between the chelating moiety and the 25 primary peptide. The purpose of inserting Aba as a spacer was to minimize any possible steric hindrance resulting from the Tc-99m complex. The synthesis of this modified peptide was one hybrid process which eliminated the multi-step, lengthy, and frequently inefficient conjugation procedure, yet provided a chelating 30 group for a strong chelation of Tc-99m. The resultant decapeptide, <sup>Gly-Pro-Arg-</sup> <sup>Pro-Pro-Aba-Gly-Gly (D)-Ala-Gly (SEQ ID NO.5)</sup> [Gly-Pro-Arg-Pro-Pro-Aba-Gly-Gly-(D) Ala-Gly] which has an expected M.W. of 850, is hereafter referred to as TP 850.

The peptide was custom synthesized (PeptidoGenic Research Co., Inc. Livermore, CA) using a Shimadzu solid phase synthesizer (Shimadzu, Columbia, MD) and separated using HFIsil, C-18, 5 micron preparative HPLC column. Ion spray mass analysis was performed using Perkin Elmer's Sciex APZ I mass spectrometer (Norwalk, CT). Using this chelating moiety and facility several peptides have previously been prepared and labeled with Tc-99m in our laboratory. (Thakur ML, Pallela VR, Consigny PM. Tc-99m-TP 1201 for imaging thromboembolism. *Radiology* 205:267P, 1997; Pallela VR, Consigny PM, Shi R, Thakur ML. Imaging vascular thrombosis with Tc-99m-TP 1300 peptide derived from active domain of thrombospondin. *J Nucl Med* 39:64P, 1998; Pallela VR, Consigny PM, Shi R, Thakur ML. Tc-99m-labeled Fibrin- $\alpha$ -chain peptide analog for imaging vascular thrombosis. *Eur J Nucl Med* 25:878, 1998; Pallela VR, Thakur ML, Consigny PM, Rao PS, Vassileva-Belnikolovska D, Shi R. Imaging thromboembolism with Tc-99m labeled thrombospondin receptor analogs TP-1201 and TP-1300. *Thrombosis Research* 93:191-202, 1999; Pallela VR, Thakur ML, Chakder S, and Rattan S. 99mTc-labeled vasoactive intestinal peptide receptor agonist: Functional studies. *J Nucl Med* 40:352-360, 1999).

20 ii) Radiolabeling and quality control:

Fifty  $\mu$ g of TP 850 was dissolved in 10  $\mu$ l 10% acetonitrile in water, then 200  $\mu$ l of 0.1 M  $\text{Na}_3\text{PO}_4$  were added, followed by 10-30 mCi Tc-99m in 200  $\mu$ l isotonic saline previously reduced with 100  $\mu$ g  $\text{SnCl}_2$  in 10  $\mu$ l of 0.05 M HCl. Lately, with a new batch of high purity  $\text{SnCl}_2$  (Sigma Chemicals, St. Louis, MO) we have been able to reduce the  $\text{SnCl}_2$  to 10  $\mu$ g. The reaction mixture was then incubated for 30 min in a boiling water bath. The product was examined by HPLC (Rainin, Emeryville, CA) using a reverse phase C-18 column and gradient solvents of 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). The gradient was such that at zero minutes solvent A was 90%, and at 30 min solvent B was 100%. The flow rate was 1 ml/min. The HPLC was equipped with a u.v. detector set at 278 nm, a 2" NaI (TI) gamma counter, and a rate meter.

iii) Stability of Tc-99m-TP 850:

5 The stability of the radiolabeled peptide at 22° C was examined by HPLC for up to 24 hrs as determined by the characteristic retention time of the radioactivity peak. The in vivo stability was examined by injecting approximately 2 mCi Tc-99m-TP 850 preparation, collecting urine 3 hrs later, and analyzing a 20 µl portion of the urine by HPLC.

10

iv) Fibrin binding:

15 The ability of Tc-99m-TP 850 to bind to rabbit, dog, and human fibrin was examined in vitro. Institutional approval was obtained to draw human blood and to perform all animal experiments. Approximately 10 ml of venous blood was obtained from a healthy human volunteer and from a normal young adult dog and a rabbit. No anticoagulating agent was added to the blood. After the blood was clotted, from each blood sample, one ml serum samples were dispensed in four separate test tubes and approximately 25 µCi of Tc-99m-TP 850 (specific activity 20 approximately 340 Ci/m mol) were added to each tube and the reagents were gently mixed. Thrombin (six i.u.) was then added to the first two test tubes and an equal volume of saline to the other two. The contents were gently mixed and allowed to incubate for 10 min at 37°C. The test tubes were then centrifuged (2000 g x 10 min), the supernatant carefully removed, and the fibrin clots in the 25 first two test tubes were washed twice with 2 ml 0.9% NaCl. Following centrifugation, the washing liquid was combined with the supernatant. Radioactivity associated with the clot and the supernatant were measured and calculated as the percent of total activity found in the compact fibrin clot.

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v) Inhibition of platelet aggregation:

Seventeen ml of venous blood from a rabbit and a dog were collected in 3 ml Acid Citrate Dextrose A (ACD A), centrifuged at 180 g for 10 min and platelet rich plasma (PRP) was separated. (Thakur ML, Coleman RE, Hoist JH, Welch M. Indium-111 labeled platelets: Studies on preparation and in vivo and in vitro functions. *Thrombosis Research* 9:345-354, 1976). Aggregation studies were performed using a Chronolog (Havertown, PA) aggregometer. For each study, increasing quantities of TP 850 and 4  $\mu$ M ADP were added to 500  $\mu$ l PRP containing approximately  $1.5 \times 10^8$  platelets, stirring at 37°C. Aggregation in the absence of TP 850 was considered 100% and IC<sub>50</sub> values were determined using the quantity of TP 850 that inhibited aggregation by 50%.

vi) Blood clearance:

All animal protocols were approved by the Institutional Animal Care and Use Committee and were strictly followed. Blood clearance of the agent was examined in adult New Zealand white rabbits weighing between 3 to 3.5 kg. Each rabbit was anesthetized by an i.m. injection of ketamine (30 mg/kg) and zylaxine (5 mg/kg). Thereafter a 23 gauge catheter was inserted in the right ear artery and connected to a leuer lock (Burron Med. Inc., Bethlehem, PA). The patency of the catheter was maintained by the administration of 6 i.u. heparin per ml of sterile 0.9% NaCl administered through the leuer lock. This catheter was used for drawing 0.5 ml blood samples in duplicate at 1,5,10,15, and 30 min, and then at 1, 2, and 3 hrs after radionuclide injection. Before each sample collection enough blood was withdrawn to replace saline, which avoided the dilution of each blood sample collected.

The marginal vein of the contralateral ear was used for injecting radioactive agents. The radioactivity in the syringe was measured before and after injection to determine the dose injected, and a suitable Tc-99m standard solution was prepared. Blood samples were then weighed, radioactivity counted, and results were

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expressed as percent injected dose per gram (% I.D./g) of blood and plotted as a function of time.

5           vii) Tissue distribution studies:

Tissue samples were harvested from three rabbits three hrs after the administration of Tc-99m-TP 850. Tissues were weighed, radioactivity associated with each tissue, and a reference standard solution of Tc-99m prepared at the time  
10 of injection was determined. Radioactivity was expressed as % injected dose/g (% I.D./g) of tissue. Results were averaged and standard deviation was determined.

viii) Inducing DVT:

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Each of the eight adult (male or female) New Zealand white rabbits, weighing between 3-3.5 kg was anesthetized as described above, the right cubital vein and/or jugular vein was exposed, and a stimulating electrode was inserted (Leadley RJ, Humphrey WR, Erickson LA, Shebuski RJ. Inhibition of thrombus formation  
20 by Endothelin-1 in canine models of arterial thrombosis. *Thrombosis and Haemostasis* 74:1583-1590, 1995). The electrode was constructed from a 26-gauge stainless steel hypodermic needle bent at a 90° angle and attached to a 30-gauge, Teflon insulated silver coated copper wire. The needle was inserted into the vessel and then gently pulled so that it was in contact with the endothelial  
25 lining of the vessel and secured in place with a flared sleeve inserted over the copper wire. The second electrode was applied to the tongue of the rabbit. The stimulating electrode was attached to the anode and the other electrode to the cathode of a power supply. A 450 µA current was then applied and 10 min later 2 mCi of Tc-99m TP 850 (specific activity approximately 510 Ci/m mol) in 2 ml  
30 0.9% solution was injected through a marginal ear vein. Radioactivity in each dose was measured before and after administration and recorded. A suitable reference solution with a known quantity of Tc-99m was also prepared. In two additional rabbits thrombus was induced by inserting a thrombin-soaked suture into

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a jugular vein 10 min prior to the administration of Tc-99m-TP 850. Serial gamma camera images of the rabbit, in the supine position, were then obtained for up to four hours, using a GE Starcam gamma camera (GE, Milwaukee, WI) coupled to a low energy parallel hole collimator. For each image a total of  
5 350,000 counts were collected.

ix) Inducing pulmonary embolism:

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Pulmonary emboli were induced in six additional rabbits. Radio opaque pulmonary emboli were prepared by drawing 0.5 to .75 ml blood, through a 23G butterfly needle inserted in the marginal ear vein, into a one ml syringe containing 15 mg tantalum powder and 6 i.u. of thrombin. The contents of the syringe were  
15 then mixed gently and a clot was allowed to form and harden for 20 min. The clot was removed from the syringe and a one cm long piece of the clot was drawn in an introducer sheath (6Fr, Pinnacle, MediTech, Watertown, MA), which was then inserted into a previously isolated jugular vein and advanced into the right atrium. The clot was then flushed from the sheath with isotonic saline. The position of the  
20 tantalum containing clots was confirmed by recording a chest x-ray of the animals before the administration of Tc-99m-TP 850 and an x-ray of the excised lungs after sacrifice. Following clot administration and the confirmation of its localization by x-ray, Tc-99m-TP 850 was injected and the rabbits were imaged as described in the previous section. Four animals with PE were allowed to recover from the  
25 surgery. Two rabbits were injected with Tc-99m-TP 850 24 hrs later and the other two were injected 48 hours later.

Upon the conclusion of imaging for PE or DVT, each rabbit was given an intravenous injection of heparin (1000 i.u.) and was then euthanized with sodium pentobarbital (100 mg/kg). A blood sample was drawn, and then the lungs and  
30 heart were excised, radiographed, and the clots were harvested. The clots and blood were weighed, radioactivity associated with them was counted, and clot/blood ratios were determined.

### Results

#### i) Peptide radiolabeling, quality control, and stability:

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The purity of the peptide as determined by HPLC analysis was > 90%. The expected M.W. of the peptide was 850, and the one observed by mass spectroscopic analysis was 849.4. The proposed structure of Tc-99m labeled TP 850 is given in Fig. 1, which shows that Tc-99m is bound to the chelating moiety with N<sub>4</sub> configuration. The Tc-99m labeling consistently produced > 95% yield. HPLC analysis indicated that > 90% of that activity was eluted in a single peak at retention time (Rt) of seven min. A small quantity (< 5%) of radioactivity was eluted at a Rt of 6.2 min and any unbound Tc-99m at a Rt of 3.5 min. An elution profile is given in Fig. 2.

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The preparations of Tc-99m-TP 850 were stable at 22°C for 24 hrs. HPLC analysis of a urine sample (Fig. 2) collected at three hrs after an injection of Tc-99m-TP 850 showed that the radioactivity elution profile was similar to that of the preparation injected, and that the retention time of the radioactivity peak in the urine sample was similar to that of the radioactivity sample injected. This suggested that the small peptide was not susceptible to a rapid in vivo proteolysis.

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#### ii) Blood clearance and tissue distribution:

The blood clearance was biphasic with a  $t_{1/2-\alpha}$  being approximately four min (20%) and  $t_{1/2-\beta}$  being approximately 13 min (80%). Examination of three hr tissue distribution of Tc-99m-TP 850 indicated that the highest radioactivity was in the kidneys ( $0.10 \pm 0.086$  % I.D./g), suggesting that the kidneys were the primary route of excretion. The liver uptake was  $0.016 \pm 0.014$  % I.D./g and intestine  $0.01 \pm 0.009$  % I.D./g. The blood uptake at this time was only  $0.007 \pm 0.004$  % I.D./g. This small proportion of radioactivity in circulating blood facilitated the imaging of vascular thrombi. Radioactivity in all other tissues was unremarkable.

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ii) Fibrin binding and inhibition of platelet aggregation:

TP 850 radioactivity associated with human, dog, and rabbit fibrin was  $42 \pm 2\%$ ,  $60 \pm 39\%$ , and  $56 \pm 2.5\%$ , respectively. The  $IC_{50}$  values for the dog and rabbit platelet aggregation inhibition were  $236 \mu M$  and  $167 \mu M$ , respectively. These data justified the use of rabbit as a model for studies with Tc-99m-TP 850.

10 iii) Imaging DVT and PE:

Although Tc-99m-TP 850 cleared rapidly from the blood, cardiac blood pool activity was detectable in all animals at all imaging times. Radioactivity in the sinus was also detectable in all animals studied. This was consistent with the results of Tc-99m-TP1201 and Tc-99m-TP1300, the activated platelet receptor specific thrombospondin analogs studied previously in our laboratory. (Pallela VR, Thakur ML, Consigny PM, Rao PS, Vassileva-Belnikolovska D, Shi R. Imaging thromboembolism with Tc-99m labeled thrombospondin receptor analogs TP-1201 and TP-1300. *Thrombosis Research* 93:191-202, 1999). Unlike these two agents, however, no Tc-99m-TP 850 radioactivity was seen either in the bone or in the bone cartilage. All fresh DVT and PE were detectable by Tc-99m-TP 850 generally within 90-120 min post-injection. Clots that formed spontaneously in surgical incision or ligated vessels were also detectable. Similarly, PE that were formed by a piece of a clot broken off or separated from a clot that was injected into the right atrium were imageable. An example is given in Fig. 3, in which an electrode-induced clot in the right forearm, two PE, one in each upper lobe of the lungs, and radioactivity accumulated in the incision was seen. In this animal, the forearm clot to blood radioactivity ratio was 12, and the PE to blood 1.3 (L), and 2.1 (R). The radioactivity associated with the clots was 0.087% I.D./g, 0.006 % I.D./g and 0.007% I.D./g, respectively.

The clot/blood ratios in the rabbits studied ranged from 1.2 to 12. Many times these clots were small and could not be easily separated without the vessel wall or adjoining fatty tissue. Similarly, tantalum was embedded in many PE.



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Consequently, the weight contributed by the additional tissue or tantalum, resulted in the low and variable radioactivity per unit weight of clots, PE, or DVT.

Fig. 4 shows an anterior image of a rabbit given Tc-99m-TP 850 one hr and 20 min previously. A clot in the right jugular vein induced by stimulating electrode and the one in the left jugular vein induced by thrombin-soaked suture were detectable. The clot to blood ratios were 6.5 and 3.7, respectively. The clot radioactivity was 0.035% I.D./g and 0.02% I.D./g. In this animal, the radioactivity was also seen in the thyroid due to 3.5% unbound free Tc-99m that was injected.

Fig. 5 shows an anterior image of a rabbit obtained at 150 min after the injection of Tc-99m-TP 850 into which thrombin-soaked suture was placed in the right jugular vein and a stimulating electrode clot was formed in the left jugular vein. Both clots were detectable with the electrode clot to blood ratio of 7.1 and the suture soaked thrombin clot/blood ratio of 3.6. Included in each suture clot was the weight of the suture itself which artificially decreased the clot/blood radioactivity ratios. The radioactivity incorporated into these clots measured 0.046% I.D./g and 0.024% I.D./g of the weight of the clot.

Fig. 6 is an anterior image of a rabbit with PE in both lungs induced 24 hrs previously. The image was positive at one hr and 15 min post-injection of Tc-99m-TP 850. Lungs were excised, imaged, and x-rayed. The location of the clots was corroborated. The clots were then retrieved, weighed, and associated radioactivity was measured. The clot to blood ratios were 6.1 for the right clot and 3.0 for the one in the left clot. The radioactivity in the clot was 0.021% I.D./ and 0.01% I.D./g.

In contrast, 48 hr old clots were neither detectable by scintigraphy nor by x-ray. This suggested that they were lysed and had disappeared. This is consistent with the high fibrinolytic activity in rabbits. (Didisheim P. Animal models useful in the study of thrombosis and antithrombotic agents. *Prog in Hemostasis and Thrombosis* Spaet TH, ed. Grune and Stratton: New York, pp. 165-197, 1976; Doolittle RF, Omcley JL, and Surgenor DM. Species differences in the interaction of thrombin and fibrinogen. *J Biol Chem* 237:3123, 1962; Gallimore MJ, Nulkar MV, and Shaw JTB. A comparative study of the inhibitors of fibrinolysis in human, dog, and rabbit blood. *Thromb Diath Haemorrh* 14:145-



In principle, external scintigraphic techniques aided by the use of a suitable radiopharmaceutical can provide hot spot images and can fulfill the need since scintigraphic techniques are non-invasive, and can scan the entire body of a patient without unreasonable inconvenience or added morbidity to the patient.

5           During the past few years, a large number of radiopharmaceuticals have been investigated as potential agents to localize DVT or PE. Since thrombi are largely composed of fibrin, platelets and other cells entrapped in the fibrin network, much attention was drawn to the use of radioiodine labeled fibrinogen and In-111 labeled platelets.

10           In many ways, radiolabeled platelets should be a simple and ideal agent, for they form a major and the most biologically active constituent of a thrombus. However, radiolabeled platelets have been less attractive largely due to their long life span (8 days) that elevated background radioactivity for several days after their administration. The slow clearance of radioactivity causes delay in diagnosis due  
15 to suboptimal lesion to background radioactivity ratios. The platelets must also be labeled in vitro which requires skilled personnel. Furthermore, in the presence of anticoagulant therapy, heparin in particular, when accretion of fresh platelets is impeded, In-111 platelet scintigraphy is less successful. An array of antibodies, the majority of them specific for IIb and IIIa glycoprotein complex on the platelet  
20 surface, have also been investigated. Success with these has been limited for a variety of reasons including the lack of specificity, unfavorable pharmacokinetics or cumbersome preparation of the agent. The pros and cons of these and other agents have been described by Knight, Thakur, and Koblik et al.

          Prompted by the advancements in science and technology of molecular  
25 biology, recent development of radioactive agents for non-invasive diagnosis of thromboembolism are centered around the use of Tc-99m labeled peptides specific for resting or activated platelets. Peptides are smaller in size and easier to produce than monoclonal antibodies. They are expected to clear more rapidly from circulation than radiolabeled proteins, less likely to induce any immunological  
30 reaction, yet in most cases they enjoy as high a receptor specificity and binding constants as the monoclonal antibodies. Because of the physical characteristic of Tc-99m, the Tc-99m labeled peptides have become even more attractive biomolecules for diagnostic imaging than antibodies labeled with In-111.



Peptides chosen for scintigraphic imaging are modified before they are labeled with a radionuclide of choice. In order to accomplish efficient radiolabeling, most commonly, the presynthesized peptides are conjugated with a metal chelating agent. This is a multi-step process in which peptide functional groups are first blocked, chelating agents are conjugated, and excess of reagents are eliminated. The functional groups from the resultant product are then deblocked, the product is separated using preparative HPLC, and the required product is identified by mass spectroscopic analysis. Not only is the procedure time-consuming, but it can also be frequently inefficient.

The hybrid peptide technique which we developed to label the peptide with Tc-99m, is simple, efficient, and eliminates the drawbacks stated above. The results of our fibrin clot binding and platelet aggregation inhibition studies support the notion that these modifications did not compromise the biological activity of the peptide. These results are consistent with previous findings using biologically active peptides.

The binding of Tc-99m-TP 850 to rabbit fibrin and its IC<sub>50</sub> value for inhibition of rabbit platelet aggregation observed in this study were high enough to justify the use of the rabbit as a model for imaging experimental clots and PE. All clots, formed by vessel wall injury, by stimulating electrode or by thrombin-soaked sutures implanted in the jugular vein, were detectable by gamma scintigraphy. In general, the clots were small and the radioactivity incorporated into them varied from 0.01% I.D./g to 0.087% I.D./g. This variability was probably due to the presence of non-radioactive tissues or tantalum that were not separated but contributed to the weight of the clots. However, neither the proportion of radioactivity incorporated into the clots, nor the variation of this proportion is uncommon in such animal experiments. Despite the relatively small proportion of radioactivity, the clots were detectable in approximately 90 min after injection.

In experiments, Tc-99m-TP 850 had considerably higher radioactivity uptake on PE than at least two activated platelet specific Tc-99m labeled peptides we had evaluated previously. With Tc-99m-TP 850, all PE were detectable except those that had lysed spontaneously at 48 hr post-placement. The disappearance of the 48 hr old clots was confirmed by the loss of x-ray opacity of these clots which

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had been impregnated with tantalum at the time of preparation. The choice of using the rabbit as a model was based upon our supportive in vitro data described previously. However, the plasminogen concentration in rabbits is greater than twice as high as in humans. The fibrinolytic activity in rabbits, therefore, is much higher and leads to a rapid dissolution of these clots.

In principle, an antifibrin agent should be more successful in imaging aged thrombi and may be less susceptible to interference by anticoagulant therapy because in such circumstances more fibrin may be exposed on the clot surface and blood flow around the clot may be greater.

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**I CLAIM:**

1. A composition having formula I or II:



wherein:

$X_1$  is from zero to twenty natural or synthetic amino acids;

P is a peptide comprising Gly Pro Arg (SEQ ID NO: 2), or an analog or fragment thereof;

$X_2$  is from zero to twenty natural or synthetic amino acids;

Z is a linker comprising one or more natural or synthetic amino acids; and

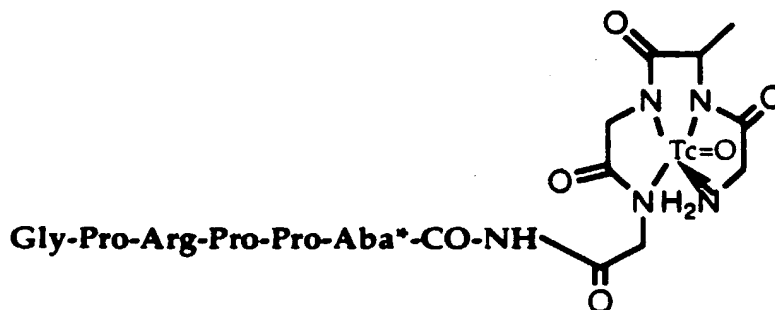
M is a radiolabeling moiety comprised of a chelating moiety capable of complexing with a selected radionuclide.

2. The composition according to Claim 1 comprising SEQ ID NO: 1.

3. The composition according to Claim 1, wherein the radiolabeling moiety is complexed to the radionuclide.

4. The composition according to Claim 3, wherein the radionuclide is technetium-99m.

5. The composition according to Claim 3 having the formula:



6. The composition according to Claim 1, wherein M comprises Gly -(D)-Ala-Gly-Gly (SEQ ID NO: 3) as a chelating moiety for a radionuclide.





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ABSTRACT

5           The present invention involves compositions for radiolabeled agents for imaging  
mammalian tissue or cells, compositions for radiolabeling agents that bind to mammalian  
tissue or cells, compositions for radiolabeling agents that bind to fibrin, and methods of  
using said compositions.

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IMAGING WITH TC-99M LABELED FIBRIN- $\alpha$ -CHAIN PEPTIDE

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GOVERNMENT RIGHTS IN THE INVENTION

This invention was made with government support under grant R41 HL  
59769-01 (MLT) awarded by the National Institutes of Health. The government has  
10 certain rights in the invention.

CROSS REFERENCE TO RELATED APPLICATIONS

15 This application claims priority under 35 U.S.C. § 119 based upon U.S.  
Provisional Patent Application No. 60/096,803 filed August 17, 1998.

FIELD OF THE INVENTION

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The present invention generally relates to the field of nuclear medicine and, more  
particularly, to compositions for radiolabeled agents for imaging mammalian tissue or cells,  
compositions for radiolabeling agents that bind to mammalian tissue or cells, compositions  
for radiolabeling agents that bind to fibrin, and methods of using said compositions.

25

BACKGROUND OF THE INVENTION

Development of radioactive agents for "hot spot" imaging of deep venous  
30 thrombosis (DVT) and pulmonary embolism (PE) has been the subject of many  
investigations for more than two decades. (Thakur ML, Coleman RE, Hoist JH,  
Welch M. Indium-111 labeled platelets: Studies on preparation and *in vivo* and *in*  
*vitro* functions. *Thrombosis Research* 9:345-354, 1976; Knight LC.

Radiopharmaceuticals for thrombus detection. *Seminars in Nucl Med* XX:52-67, 1990; Koblik PD, DeNardo GL, Berger HJ. Current status of Immunoscintigraphy in the detection of thrombosis and thromboembolism. *Seminars in Nucl Med* XIX:221-231, 1989; Thakur ML. Potential of radiolabeled antiplatelet antibodies in the detection of vascular thrombi. In: S.C. Srivastava, ed. *Radiolabeled monoclonal antibodies for imaging and therapy*. Plenum Publishing Co., NATO ASI, series 152, 1988; Thakur ML. Scintigraphic imaging of venous thrombosis: A state of the art. *Thrombotic and Hematologic Disorders* 5:29-36, 1992). One approach to "hot spot" imaging has been to radiolabel platelets, which form a major biochemically active constituent of a thrombus. A large number of agents, therefore, have been evaluated that would target platelets on the assumption that radiolabeled platelets will accrete on an occult thrombus and thereby facilitate its detection by external scintigraphy. Platelets have been labeled *in vitro* using such agents as In-111-oxine which internalizes and binds to platelet cytoplasmic components. (Thakur ML et al., 1976). Platelets have also been labeled *in vivo* using radiolabeled proteins or peptides that are specific for platelet surface glycoprotein complex IIb-IIIa (Knight LC, 1990; Koblik PD et al., 1989; Thakur ML, 1988; Thakur ML, 1992; Knight LC, Radcliffe R, Maurer AH, Rodwell JD, Alvarez VL. Thrombus imaging with Tc-99m synthetic peptides based upon the binding domain of a monoclonal antibody to activated platelets. *J Nucl Med* 35:282-288, 1994; Knight LC, Maurer AH, Romano JE. Comparison of Iodine-123-Disintegrins for Imaging Thrombi and Emboli in a Canine Model. *J Nucl Med* 37:476-482, 1996; Pearson DA, Lister-James J, McBride WJ, Wilson DM, Martel LJ, Civitello ER, Dean RT. Thrombus imaging using Tc-99m labeled high potency GPIIb./IIIa receptor antagonist. Chemistry and initial biological studies. *J Med Chem* 39:1372-1382, 1996; Lister-James J, Vallabhajosula S, Moyer BR, Pearson DA, McBride BJ, De Rosch MA, Bush LR, Machac J, Dean RT. Pre-Clinical Evaluation of Technetium-99m platelet receptor-binding platelet. *J Nucl Med* 38:105-111, 1997; Line BR, Crane P, Lazewatsky J, Barrett JA, Cloutier D, Kagan M, Lukasiewicz R, Holmes RA. Phase I trial of DMP444, a new thrombus imaging agent. *J Nucl Med* 37:117P, 1996; Barrett JA, Crocker AC, Damphousse DJ, Heminway SJ, Liu S, Edwards DS, Lazewatsky JL, Kagan M, Mazaika TJ, Carroll TR. Biological evaluation of 99mTc cyclic glycoprotein IIb/IIIa receptor

antagonists in the canine arteriovenous shunt and deep vein thrombosis models: Effects of chelators on biological properties of [99mTc]chelator—peptide conjugates. *Bioconjugate Chem* 7:203-208, 1996). Despite the success achieved with these agents in experimental animals and in limited human subjects, only one  
5 agent, AcuTect, the Tc-99m labeled peptide P-280, has recently been approved for clinical use. AcuTect is expected to detect acute but not chronic venous thrombosis (AcuTect. Diatide, Inc. *J Nucl Med* 39(10):19N, 1998) or pulmonary embolism, which may harbor activated platelets only sparingly.

A second approach to “hot spot” imaging has been to radiolabel proteins  
10 involved in clot formation. During the vessel wall injury, coagulation proteins are activated sequentially and generate the enzyme thrombin. Thrombin cleaves plasma fibrinogen into fibrin monomers, which then polymerize around the platelets and hold them in place as a clot. Fibrin therefore remains an integral part of DVT, fresh or old, and embolized in the lungs or elsewhere in the body. It is  
15 primarily for these reasons that I-125-fibrinogen enjoyed popularity for external detection of DVT for a long time (Knight LC, 1990; Koblik PD et al., 1989; Thakur ML, 1992). However, it is no longer available commercially. Iodine-123-fibrinogen and many antifibrin monoclonal antibodies labeled with various radionuclides have also been evaluated. (Koblik PD et al., 1989). However, for  
20 many reasons such as the long circulation times or poor image quality, agents other than I-125-fibrinogen did not make it into common nuclear medicine practice.

A third approach to “hot spot” imaging of DVT and PE is to radiolabel antifibrin peptides. The feasibility of this approach has not been previously  
25 investigated. One peptide of particular interest is the N-terminus tripeptide, H-Gly-Pro-Arg-OH, of fibrin- $\alpha$ -chain, which was reported by Laudano and Doolittle to be an inhibitor of fibrinogen/thrombin clotting. (Laudano AP, Doolittle RF. Synthetic peptide derivatives that bind to fibrinogen and prevent the polymerization of fibrin monomers. *Proc Natl Acad Sci* 75:3085-3089, 1978). The investigators observed  
30 that H-Gly-Pro-Arg-Pro-OH analog of the tripeptide was an even more potent inhibitor of fibrinogen/thrombin clot by binding to C-terminus portion of the  $\gamma$ -chain of fibrin and preventing fibrin polymerization. More recently, Kawasaki et al prepared several more analogs and found that a pentapeptide, H-Gly-Pro-Arg-Pro-



that of the urine sample collected from the rabbit 3 hrs later. Note that the major proportion of the radioactivity eluted in the urine has the retention time (Rt) similar to that in the sample injected. The radioactivity at Rt 4 is unbound Tc-99m. The small radioactivity peaks at Rt 6.2 and 9.08 are considered as  
5 impurities in the sample. The quantity of the peptide injected was small and was not detectable at 280 nm.

**Fig. 3.** An anterior image of a rabbit obtained at 3 hr post-injection. A small thrombus induced by stimulating electrode in the right arm (arrowhead) and PE in both upper lobes of the lungs (long arrows) are detectable. Also seen in the  
10 right side of the neck (short arrows) is radioactivity accumulated in the incision. The radioactivity in the heart and sinus can be seen.

**Fig. 4.** An anterior image of a rabbit obtained at 1hr 20 min post-injection. A clot induced by stimulating electrode (clot/blood = 6.5) and the one by thrombine-soaked suture (clot/blood = 3.7) are detectable. In addition,  
15 radioactivity in the heart, thyroid, and paranasal sinuses can be seen. Free Tc-99m in preparation was approximately 3%.

**Fig. 5.** Anterior gamma camera images of a rabbit which was injected with 2 mCi Tc-99m-TP850 2 hr and 30 min previously. A clot due to thrombine-soaked suture in the right (arrow) jugular vein and due to stimulating electrode in  
20 the left (arrow) are detectable. The activity due to some free Tc-99m in the thyroid can also be seen. As stated in the text, the electrode clot had 7.1 times more Tc-99m than that in the equal weight of blood and the thrombin clot had 3.6 times more Tc-99m than in the blood. The lower part of the radioactivity is in the heart.

**Fig. 6.** A composite of three images from one rabbit, in which PE was induced 24 hr prior to the i.v. administration of 2.4 mCi of Tc-99m-TP 850. The scintiphoto in the left panel of the figure was obtained at 1 hr 15 min post-injection in the anterior position. It shows abnormal accumulation of radioactivity in both lungs (arrow). A clot formed spontaneously in the left neck where the incision was  
30 made for the placement of the PE introducer sheath is also seen in the scintiphoto given in the left panel of the figure. At the conclusion of in vivo scintigraphy, the heart and lungs were excised, spread for clarity, and then imaged under a gamma camera, as well as x-rayed. The x-ray image (center panel) shows a tantalum

mixed clot in the left lung which corresponds to the shape of a clot seen in the left lung (anterior scintiphoto in the left panel of the figure), as well as to the left lung clot seen in the gamma camera image of the excised lungs and heart given in the right panel of the figure. The clot seen in the right lung, in both in vivo (arrow, 5 left panel) and ex vivo (arrow, right panel) images is not seen by x-ray (center panel) because it is free of tantalum. This indicates that this piece of clot may have formed without tantalum in it and lodged in the right lung. Both lung clots were separated, weighed, and counted for radioactivity. The clot in the left lung had three times more and the one in the right lung had 6.1 times more activity than 10 in the unit weight of blood. This clot in the neck had 3.2 times more activity than in the unit weight of blood. Residual blood radioactivity in the heart (H) can also be seen in the right panel of the figure.

## **DETAILED DESCRIPTION**

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### **Materials and Methods**

#### **i) Preparation of peptide**

For this study, a group of four amino acids, Gly-(D)-Ala-Gly-Gly- 20 (GAGG) was chosen as a chelating moiety. Through their NH<sub>2</sub> groups these peptides provide an N<sub>4</sub> configuration for a strong chelation of Tc-99m. Rather than the conventional post-synthesis conjugation, the tetrapeptide chelating moiety permitted the modification of the primary peptide at the C terminus during the synthesis. Furthermore, during the synthesis, an additional amino acid, Aba (4- 25 aminobutyric acid), was inserted as a spacer between the chelating moiety and the primary peptide. The purpose of inserting Aba as a spacer was to minimize any possible steric hindrance resulting from the Tc-99m complex. The synthesis of this modified peptide was one hybrid process which eliminated the multi-step, lengthy, and frequently inefficient conjugation procedure, yet provided a chelating 30 group for a strong chelation of Tc-99m. The resultant decapeptide, Gly-Pro-Arg-Pro-Pro-Aba-Gly-Gly-(D) Ala-Gly which has an expected M.W. of 850, is hereafter referred to as TP 850.

The peptide was custom synthesized (PeptidoGenic Research Co., Inc. Livermore, CA) using a Shimadzu solid phase synthesizer (Shimadzu, Columbia, MD) and separated using HFIsil, C-18, 5 micron preparative HPLC column. Ion spray mass analysis was performed using Perkin Elmer's Sciex APZ I mass spectrometer (Norwalk, CT). Using this chelating moiety and facility several peptides have previously been prepared and labeled with Tc-99m in our laboratory. (Thakur ML, Pallela VR, Consigny PM. Tc-99m-TP 1201 for imaging thromboembolism. *Radiology* 205:267P, 1997; Pallela VR, Consigny PM, Shi R, Thakur ML. Imaging vascular thrombosis with Tc-99m-TP 1300 peptide derived from active domain of thrombospondin. *J Nucl Med* 39:64P, 1998; Pallela VR, Consigny PM, Shi R, Thakur ML. Tc-99m-labeled Fibrin- $\alpha$ -chain peptide analog for imaging vascular thrombosis. *Eur J Nucl Med* 25:878, 1998; Pallela VR, Thakur ML, Consigny PM, Rao PS, Vassileva-Belnikolovska D, Shi R. Imaging thromboembolism with Tc-99m labeled thrombospondin receptor analogs TP-1201 and TP-1300. *Thrombosis Research* 93:191-202, 1999; Pallela VR, Thakur ML, Chakder S, and Rattan S. 99mTc-labeled vasoactive intestinal peptide receptor agonist: Functional studies. *J Nucl Med* 40:352-360, 1999).

ii) Radiolabeling and quality control:

Fifty  $\mu$ g of TP 850 was dissolved in 10  $\mu$ l 10% acetonitrile in water, then 200  $\mu$ l of 0.1 M  $\text{Na}_3\text{PO}_4$  were added, followed by 10-30 mCi Tc-99m in 200  $\mu$ l isotonic saline previously reduced with 100  $\mu$ g  $\text{SnCl}_2$  in 10  $\mu$ l of 0.05 M HCl. Lately, with a new batch of high purity  $\text{SnCl}_2$  (Sigma Chemicals, St. Louis, MO) we have been able to reduce the  $\text{SnCl}_2$  to 10  $\mu$ g. The reaction mixture was then incubated for 30 min in a boiling water bath. The product was examined by HPLC (Rainin, Emeryville, CA) using a reverse phase C-18 column and gradient solvents of 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). The gradient was such that at zero minutes solvent A was 90%, and at 30 min solvent B was 100%. The flow rate was 1 ml/min. The HPLC was equipped with a u.v. detector set at 278 nm, a 2" NaI (TI) gamma counter, and a rate meter.



iii) Stability of Tc-99m-TP 850:

5 The stability of the radiolabeled peptide at 22° C was examined by HPLC for up to 24 hrs as determined by the characteristic retention time of the radioactivity peak. The in vivo stability was examined by injecting approximately 2 mCi Tc-99m-TP 850 preparation, collecting urine 3 hrs later, and analyzing a 20 µl portion of the urine by HPLC.

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iv) Fibrin binding:

The ability of Tc-99m-TP 850 to bind to rabbit, dog, and human fibrin was examined in vitro. Institutional approval was obtained to draw human blood and to perform all animal experiments. Approximately 10 ml of venous blood was obtained from a healthy human volunteer and from a normal young adult dog and a rabbit. No anticoagulating agent was added to the blood. After the blood was clotted, from each blood sample, one ml serum samples were dispensed in four separate test tubes and approximately 25 µCi of Tc-99m-TP 850 (specific activity approximately 340 Ci/m mol) were added to each tube and the reagents were gently mixed. Thrombin (six i.u.) was then added to the first two test tubes and an equal volume of saline to the other two. The contents were gently mixed and allowed to incubate for 10 min at 37°C. The test tubes were then centrifuged (2000 g x 10 min), the supernatant carefully removed, and the fibrin clots in the first two test tubes were washed twice with 2 ml 0.9% NaCl. Following centrifugation, the washing liquid was combined with the supernatant. Radioactivity associated with the clot and the supernatant were measured and calculated as the percent of total activity found in the compact fibrin clot.

30

v) Inhibition of platelet aggregation:

Seventeen ml of venous blood from a rabbit and a dog were collected in 3 ml Acid Citrate Dextrose A (ACD A), centrifuged at 180 g for 10 min and platelet rich plasma (PRP) was separated. (Thakur ML, Coleman RE, Hoist JH, Welch M. Indium-111 labeled platelets: Studies on preparation and in vivo and in vitro functions. *Thrombosis Research* 9:345-354, 1976). Aggregation studies were performed using a Chronolog (Havertown, PA) aggregometer. For each study, increasing quantities of TP 850 and 4  $\mu$ M ADP were added to 500  $\mu$ l PRP containing approximately  $1.5 \times 10^8$  platelets, stirring at 37°C. Aggregation in the absence of TP 850 was considered 100% and IC<sub>50</sub> values were determined using the quantity of TP 850 that inhibited aggregation by 50%.

vi) Blood clearance:

All animal protocols were approved by the Institutional Animal Care and Use Committee and were strictly followed. Blood clearance of the agent was examined in adult New Zealand white rabbits weighing between 3 to 3.5 kg. Each rabbit was anesthetized by an i.m. injection of ketamine (30 mg/kg) and zylaxine (5 mg/kg). Thereafter a 23 gauge catheter was inserted in the right ear artery and connected to a leuer lock (Burron Med. Inc., Bethlehem, PA). The patency of the catheter was maintained by the administration of 6 i.u. heparin per ml of sterile 0.9% NaCl administered through the leuer lock. This catheter was used for drawing 0.5 ml blood samples in duplicate at 1,5,10,15, and 30 min, and then at 1, 2, and 3 hrs after radionuclide injection. Before each sample collection enough blood was withdrawn to replace saline, which avoided the dilution of each blood sample collected.

The marginal vein of the contralateral ear was used for injecting radioactive agents. The radioactivity in the syringe was measured before and after injection to determine the dose injected, and a suitable Tc-99m standard solution was prepared. Blood samples were then weighed, radioactivity counted, and results were

expressed as percent injected dose per gram (% I.D./g) of blood and plotted as a function of time.

5           vii) Tissue distribution studies:

Tissue samples were harvested from three rabbits three hrs after the administration of Tc-99m-TP 850. Tissues were weighed, radioactivity associated with each tissue, and a reference standard solution of Tc-99m prepared at the time  
10 of injection was determined. Radioactivity was expressed as % injected dose/g (% I.D./g) of tissue. Results were averaged and standard deviation was determined.

viii) Inducing DVT:

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Each of the eight adult (male or female) New Zealand white rabbits, weighing between 3-3.5 kg was anesthetized as described above, the right cubital vein and/or jugular vein was exposed, and a stimulating electrode was inserted (Leadley RJ, Humphrey WR, Erickson LA, Shebuski RJ. Inhibition of thrombus formation  
20 by Endothelin-1 in canine models of arterial thrombosis. *Thrombosis and Haemostasis* 74:1583-1590, 1995). The electrode was constructed from a 26-gauge stainless steel hypodermic needle bent at a 90° angle and attached to a 30-gauge, Teflon insulated silver coated copper wire. The needle was inserted into the vessel and then gently pulled so that it was in contact with the endothelial  
25 lining of the vessel and secured in place with a flared sleeve inserted over the copper wire. The second electrode was applied to the tongue of the rabbit. The stimulating electrode was attached to the anode and the other electrode to the cathode of a power supply. A 450  $\mu$ A current was then applied and 10 min later 2 mCi of Tc-99m TP 850 (specific activity approximately 510 Ci/m mol) in 2 ml  
30 0.9% solution was injected through a marginal ear vein. Radioactivity in each dose was measured before and after administration and recorded. A suitable reference solution with a known quantity of Tc-99m was also prepared. In two additional rabbits thrombus was induced by inserting a thrombin-soaked suture into

a jugular vein 10 min prior to the administration of Tc-99m-TP 850. Serial gamma camera images of the rabbit, in the supine position, were then obtained for up to four hours, using a GE Starcam gamma camera (GE, Milwaukee, WI) coupled to a low energy parallel hole collimator. For each image a total of  
5 350,000 counts were collected.

ix) Inducing pulmonary embolism:

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Pulmonary emboli were induced in six additional rabbits. Radio opaque pulmonary emboli were prepared by drawing 0.5 to .75 ml blood, through a 23G butterfly needle inserted in the marginal ear vein, into a one ml syringe containing 15 mg tantalum powder and 6 i.u. of thrombin. The contents of the syringe were  
15 then mixed gently and a clot was allowed to form and harden for 20 min. The clot was removed from the syringe and a one cm long piece of the clot was drawn in an introducer sheath (6Fr, Pinnacle, MediTech, Watertown, MA), which was then inserted into a previously isolated jugular vein and advanced into the right atrium. The clot was then flushed from the sheath with isotonic saline. The position of the  
20 tantalum containing clots was confirmed by recording a chest x-ray of the animals before the administration of Tc-99m-TP 850 and an x-ray of the excised lungs after sacrifice. Following clot administration and the confirmation of its localization by x-ray, Tc-99m-TP 850 was injected and the rabbits were imaged as described in the previous section. Four animals with PE were allowed to recover from the  
25 surgery. Two rabbits were injected with Tc-99m-TP 850 24 hrs later and the other two were injected 48 hours later.

Upon the conclusion of imaging for PE or DVT, each rabbit was given an intravenous injection of heparin (1000 i.u.) and was then euthanized with sodium pentobarbital (100 mg/kg). A blood sample was drawn, and then the lungs and  
30 heart were excised, radiographed, and the clots were harvested. The clots and blood were weighed, radioactivity associated with them was counted, and clot/blood ratios were determined.

### Results

#### i) Peptide radiolabeling, quality control, and stability:

5           The purity of the peptide as determined by HPLC analysis was > 90%. The expected M.W. of the peptide was 850, and the one observed by mass spectroscopic analysis was 849.4. The proposed structure of Tc-99m labeled TP 850 is given in Fig. 1, which shows that Tc-99m is bound to the chelating moiety with N<sub>4</sub> configuration. The Tc-99m labeling consistently produced > 95% yield. HPLC analysis indicated that > 90% of that activity was eluted in a single peak at retention time (Rt) of seven min. A small quantity (< 5%) of radioactivity was eluted at a Rt of 6.2 min and any unbound Tc-99m at a Rt of 3.5 min. An elution profile is given in Fig. 2.

15           The preparations of Tc-99m-TP 850 were stable at 22°C for 24 hrs. HPLC analysis of a urine sample (Fig. 2) collected at three hrs after an injection of Tc-99m-TP 850 showed that the radioactivity elution profile was similar to that of the preparation injected, and that the retention time of the radioactivity peak in the urine sample was similar to that of the radioactivity sample injected. This suggested that the small peptide was not susceptible to a rapid in vivo proteolysis.

#### ii) Blood clearance and tissue distribution:

25           The blood clearance was biphasic with a  $t_{1/2-\alpha}$  being approximately four min (20%) and  $t_{1/2-\beta}$  being approximately 13 min (80%). Examination of three hr tissue distribution of Tc-99m-TP 850 indicated that the highest radioactivity was in the kidneys ( $0.10 \pm 0.086$  % I.D./g), suggesting that the kidneys were the primary route of excretion. The liver uptake was  $0.016 \pm 0.014$  % I.D./g and intestine  $0.01 \pm 0.009$  % I.D./g. The blood uptake at this time was only  $0.007 \pm 0.004$  % I.D./g. This small proportion of radioactivity in circulating blood facilitated the imaging of vascular thrombi. Radioactivity in all other tissues was unremarkable.

ii) Fibrin binding and inhibition of platelet aggregation:

TP 850 radioactivity associated with human, dog, and rabbit fibrin was  $42 \pm 2\%$ ,  $60 \pm 39\%$ , and  $56 \pm 2.5\%$ , respectively. The  $IC_{50}$  values for the dog and rabbit platelet aggregation inhibition were  $236 \mu\text{M}$  and  $167 \mu\text{M}$ , respectively. These data justified the use of rabbit as a model for studies with Tc-99m-TP 850.

10      iii) Imaging DVT and PE:

Although Tc-99m-TP 850 cleared rapidly from the blood, cardiac blood pool activity was detectable in all animals at all imaging times. Radioactivity in the sinus was also detectable in all animals studied. This was consistent with the results of Tc-99m-TP1201 and Tc-99m-TP1300, the activated platelet receptor specific thrombospondin analogs studied previously in our laboratory. (Pallela VR, Thakur ML, Consigny PM, Rao PS, Vassileva-Belnikolovska D, Shi R. Imaging thromboembolism with Tc-99m labeled thrombospondin receptor analogs TP-1201 and TP-1300. *Thrombosis Research* 93:191-202, 1999). Unlike these two agents, however, no Tc-99m-TP 850 radioactivity was seen either in the bone or in the bone cartilage. All fresh DVT and PE were detectable by Tc-99m-TP 850 generally within 90-120 min post-injection. Clots that formed spontaneously in surgical incision or ligated vessels were also detectable. Similarly, PE that were formed by a piece of a clot broken off or separated from a clot that was injected into the right atrium were imageable. An example is given in Fig. 3, in which an electrode-induced clot in the right forearm, two PE, one in each upper lobe of the lungs, and radioactivity accumulated in the incision was seen. In this animal, the forearm clot to blood radioactivity ratio was 12, and the PE to blood 1.3 (L), and 2.1 (R). The radioactivity associated with the clots was 0.087% I.D./g, 0.006 % I.D./g and 0.007% I.D./g, respectively.

The clot/blood ratios in the rabbits studied ranged from 1.2 to 12. Many times these clots were small and could not be easily separated without the vessel wall or adjoining fatty tissue. Similarly, tantalum was embedded in many PE.

Consequently, the weight contributed by the additional tissue or tantalum, resulted in the low and variable radioactivity per unit weight of clots, PE, or DVT.

Fig. 4 shows an anterior image of a rabbit given Tc-99m-TP 850 one hr and 20 min previously. A clot in the right jugular vein induced by stimulating electrode and the one in the left jugular vein induced by thrombin-soaked suture were detectable. The clot to blood ratios were 6.5 and 3.7, respectively. The clot radioactivity was 0.035% I.D./g and 0.02% I.D./g. In this animal, the radioactivity was also seen in the thyroid due to 3.5% unbound free Tc-99m that was injected.

Fig. 5 shows an anterior image of a rabbit obtained at 150 min after the injection of Tc-99m-TP 850 into which thrombin-soaked suture was placed in the right jugular vein and a stimulating electrode clot was formed in the left jugular vein. Both clots were detectable with the electrode clot to blood ratio of 7.1 and the suture soaked thrombin clot/blood ratio of 3.6. Included in each suture clot was the weight of the suture itself which artificially decreased the clot/blood radioactivity ratios. The radioactivity incorporated into these clots measured 0.046% I.D./g and 0.024% I.D./g of the weight of the clot.

Fig. 6 is an anterior image of a rabbit with PE in both lungs induced 24 hrs previously. The image was positive at one hr and 15 min post-injection of Tc-99m-TP 850. Lungs were excised, imaged, and x-rayed. The location of the clots was corroborated. The clots were then retrieved, weighed, and associated radioactivity was measured. The clot to blood ratios were 6.1 for the right clot and 3.0 for the one in the left clot. The radioactivity in the clot was 0.021% I.D./ and 0.01% I.D./g.

In contrast, 48 hr old clots were neither detectable by scintigraphy nor by x-ray. This suggested that they were lysed and had disappeared. This is consistent with the high fibrinolytic activity in rabbits. (Didisheim P. Animal models useful in the study of thrombosis and antithrombotic agents. *Prog in Hemostasis and Thrombosis* Spaet TH, ed. Grune and Stratton: New York, pp. 165-197, 1976; Doolittle RF, Omcley JL, and Surgenor DM. Species differences in the interaction of thrombin and fibrinogen. *J Biol Chem* 237:3123, 1962; Gallimore MJ, Nulkar MV, and Shaw JTB. A comparative study of the inhibitors of fibrinolysis in human, dog, and rabbit blood. *Thromb Diath Haemorrh* 14:145-





In principle, external scintigraphic techniques aided by the use of a suitable radiopharmaceutical can provide hot spot images and can fulfill the need since scintigraphic techniques are non-invasive, and can scan the entire body of a patient without unreasonable inconvenience or added morbidity to the patient.

During the past few years, a large number of radiopharmaceuticals have been investigated as potential agents to localize DVT or PE. Since thrombi are largely composed of fibrin, platelets and other cells entrapped in the fibrin network, much attention was drawn to the use of radioiodine labeled fibrinogen and In-111 labeled platelets.

In many ways, radiolabeled platelets should be a simple and ideal agent, for they form a major and the most biologically active constituent of a thrombus. However, radiolabeled platelets have been less attractive largely due to their long life span (8 days) that elevated background radioactivity for several days after their administration. The slow clearance of radioactivity causes delay in diagnosis due to suboptimal lesion to background radioactivity ratios. The platelets must also be labeled in vitro which requires skilled personnel. Furthermore, in the presence of anticoagulant therapy, heparin in particular, when accretion of fresh platelets is impeded, In-111 platelet scintigraphy is less successful. An array of antibodies, the majority of them specific for IIb and IIIa glycoprotein complex on the platelet surface, have also been investigated. Success with these has been limited for a variety of reasons including the lack of specificity, unfavorable pharmacokinetics or cumbersome preparation of the agent. The pros and cons of these and other agents have been described by Knight, Thakur, and Koblik et al.

Prompted by the advancements in science and technology of molecular biology, recent development of radioactive agents for non-invasive diagnosis of thromboembolism are centered around the use of Tc-99m labeled peptides specific for resting or activated platelets. Peptides are smaller in size and easier to produce than monoclonal antibodies. They are expected to clear more rapidly from circulation than radiolabeled proteins, less likely to induce any immunological reaction, yet in most cases they enjoy as high a receptor specificity and binding constants as the monoclonal antibodies. Because of the physical characteristic of Tc-99m, the Tc-99m labeled peptides have become even more attractive biomolecules for diagnostic imaging than antibodies labeled with In-111.

Technetium-99m is easy to obtain worldwide, inexpensive, and decays with gamma ray energy (140 KeV, 90%) that can be efficiently detected by gamma cameras, planar or tomographic. It has a half-life (6 hr) that is long enough to perform examinations before excessive radioactive decay has occurred, yet not too long to persist in the body long after the examinations have been carried out and to give excessive radiation dose to the subject.

All of the peptides evaluated thus far are specific for platelet glycoprotein receptor complex IIb IIIa. Among them, one peptide, Tc-99m-P280 was recently approved by the FDA under the trade name AcuTect. As per the manufacturer's description, the peptide is expected to detect only acute thrombi but not old clots or PE. A primary reason for this is physiologic, in that fresh platelets, to which AcuTect may bind in vivo, seldom accumulate in chronic clots or PE. A different approach to the problem is therefore necessary that will permit imaging of DVT as well as PE.

The coagulation process described earlier generates fibrin monomers that form a substantial part of a clot. The actual quantity of fibrin content may vary from clot to clot, but generally it is expected to be the same as the fibrinogen of the blood which in most adults is as high as five grams per 100 grams of plasma proteins. Since fibrin exists on both the surface and within clots that are forming or dissolving, the development of Tc-99m labeled peptide, specific for fibrin is appealing. Such agents, in principle, can target fibrin at any stage or state of a clot and reliably image it. For this purpose, one peptide of particular interest is the N-terminus fibrin  $\alpha$ -chain peptide, H-Gly-Pro-Arg-OH, which was reported by Laudano and Doolittle to be an inhibitor of fibrinogen/ thrombin clotting (13). The  $\alpha$ -chain of fibrin begins with the same tripeptide sequence in many animal species as well as in humans. These investigators observed that H-Gly-Pro-Arg-Pro-OH analog of the tripeptide was an even more potent inhibitor of fibrinogen/thrombin clotting than the tripeptide itself since it also bound to C-terminus portion of the  $\gamma$ -chain of fibrin and prevented fibrin polymerization. More recently, Kawasaki, et al, prepared several more analogs and found that a pentapeptide, H -Gly-Pro-Arg-Pro-Pro-OH had the highest fibrinogen/thrombin clot inhibiting activity.

Peptides chosen for scintigraphic imaging are modified before they are labeled with a radionuclide of choice. In order to accomplish efficient radiolabeling, most commonly, the presynthesized peptides are conjugated with a metal chelating agent. This is a multi-step process in which peptide functional groups are first blocked, chelating agents are conjugated, and excess of reagents are eliminated. The functional groups from the resultant product are then deblocked, the product is separated using preparative HPLC, and the required product is identified by mass spectroscopic analysis. Not only is the procedure time-consuming, but it can also be frequently inefficient.

The hybrid peptide technique which we developed to label the peptide with Tc-99m, is simple, efficient, and eliminates the drawbacks stated above. The results of our fibrin clot binding and platelet aggregation inhibition studies support the notion that these modifications did not compromise the biological activity of the peptide. These results are consistent with previous findings using biologically active peptides.

The binding of Tc-99m-TP 850 to rabbit fibrin and its  $IC_{50}$  value for inhibition of rabbit platelet aggregation observed in this study were high enough to justify the use of the rabbit as a model for imaging experimental clots and PE. All clots, formed by vessel wall injury, by stimulating electrode or by thrombin-soaked sutures implanted in the jugular vein, were detectable by gamma scintigraphy. In general, the clots were small and the radioactivity incorporated into them varied from 0.01% I.D./g to 0.087% I.D./g. This variability was probably due to the presence of non-radioactive tissues or tantalum that were not separated but contributed to the weight of the clots. However, neither the proportion of radioactivity incorporated into the clots, nor the variation of this proportion is uncommon in such animal experiments. Despite the relatively small proportion of radioactivity, the clots were detectable in approximately 90 min after injection.

In experiments, Tc-99m-TP 850 had considerably higher radioactivity uptake on PE than at least two activated platelet specific Tc-99m labeled peptides we had evaluated previously. With Tc-99m-TP 850, all PE were detectable except those that had lysed spontaneously at 48 hr post-placement. The disappearance of the 48 hr old clots was confirmed by the loss of x-ray opacity of these clots which

had been impregnated with tantalum at the time of preparation. The choice of using the rabbit as a model was based upon our supportive in vitro data described previously. However, the plasminogen concentration in rabbits is greater than twice as high as in humans. The fibrinolytic activity in rabbits, therefore, is much higher and leads to a rapid dissolution of these clots.

In principle, an antifibrin agent should be more successful in imaging aged thrombi and may be less susceptible to interference by anticoagulant therapy because in such circumstances more fibrin may be exposed on the clot surface and blood flow around the clot may be greater.

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**I CLAIM:**

1. A composition having formula I or II:



wherein:

$X_1$  is from zero to twenty natural or synthetic amino acids;

P is a peptide comprising Gly Pro Arg (SEQ ID NO: 2), or an analog or fragment thereof;

$X_2$  is from zero to twenty natural or synthetic amino acids;

Z is a linker comprising one or more natural or synthetic amino acids; and

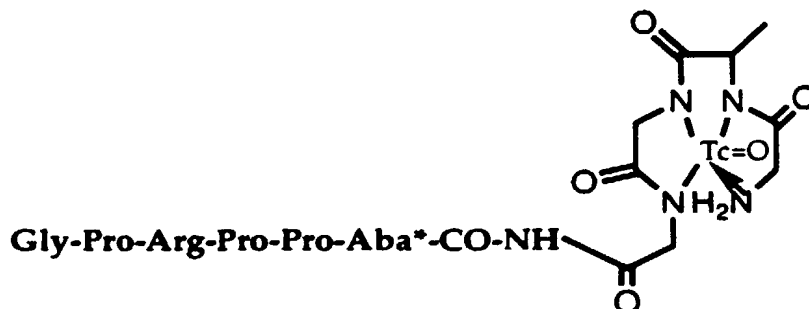
M is a radiolabeling moiety comprised of a chelating moiety capable of complexing with a selected radionuclide.

2. The composition according to Claim 1 comprising SEQ ID NO: 1.

3. The composition according to Claim 1, wherein the radiolabeling moiety is complexed to the radionuclide.

4. The composition according to Claim 3, wherein the radionuclide is technetium-99m.

5. The composition according to Claim 3 having the formula:



6. The composition according to Claim 1, wherein M comprises Gly -(D)-Ala-Gly-Gly (SEQ ID NO: 3) as a chelating moiety for a radionuclide.



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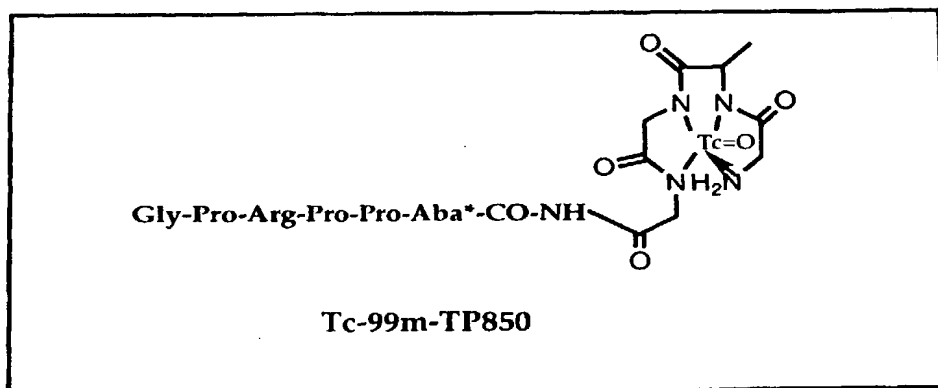
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<p>(21) International Application Number: PCT/US99/19011</p> <p>(22) International Filing Date: 17 August 1999 (17.08.99)</p> <p>(30) Priority Data: 60/096,803 17 August 1998 (17.08.98) US</p> <p>(71) Applicant: THOMAS JEFFERSON UNIVERSITY [US/US]; 1020 Walnut Street, Philadelphia, PA 19107 (US).</p> <p>(72) Inventor: THAKUR, Madhukar (Mathew), L.; 44 Lakeview Drive, Cherry Hill, NJ 08003 (US).</p> <p>(74) Agent: WEBER, Clifford, Kent; Thomas Jefferson University, Office of University Counsel, Suite 620, 1020 Walnut Street, Philadelphia, PA 19107-5587 (US).</p>		<p>(81) Designated States: CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p><b>Published</b> <i>With international search report.</i></p> <p>(88) Date of publication of the international search report: 11 May 2000 (11.05.00)</p>

(54) Title: IMAGING WITH TC-99M LABELED FIBRIN-ALPHA-CHAIN PEPTIDE



(57) Abstract

The present invention involves compositions for radiolabeled agents for imaging mammalian tissue or cells, compositions for radiolabeling agents that bind to mammalian tissue or cells, compositions for radiolabeling agents that bind to fibrin, and methods of using said compositions.

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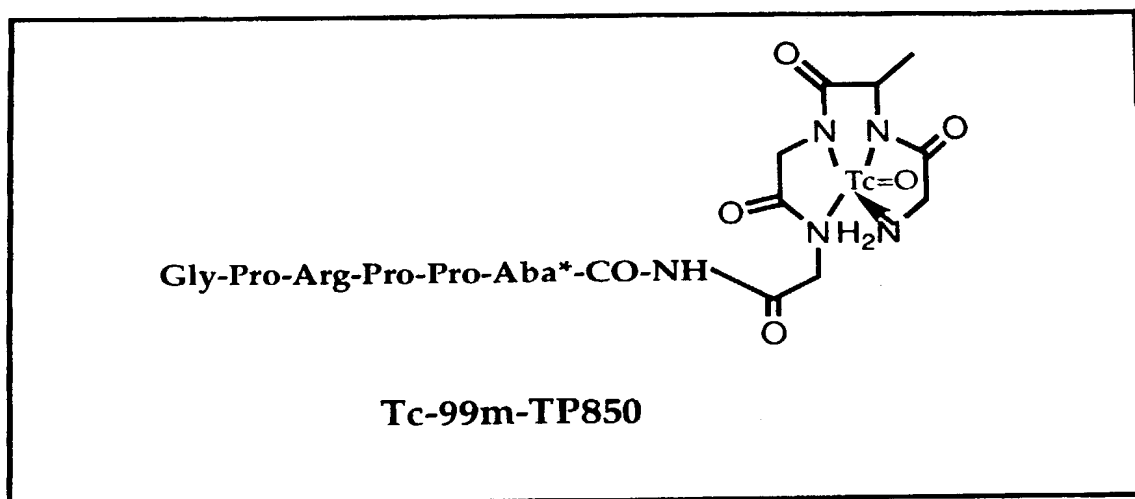


Fig. 1



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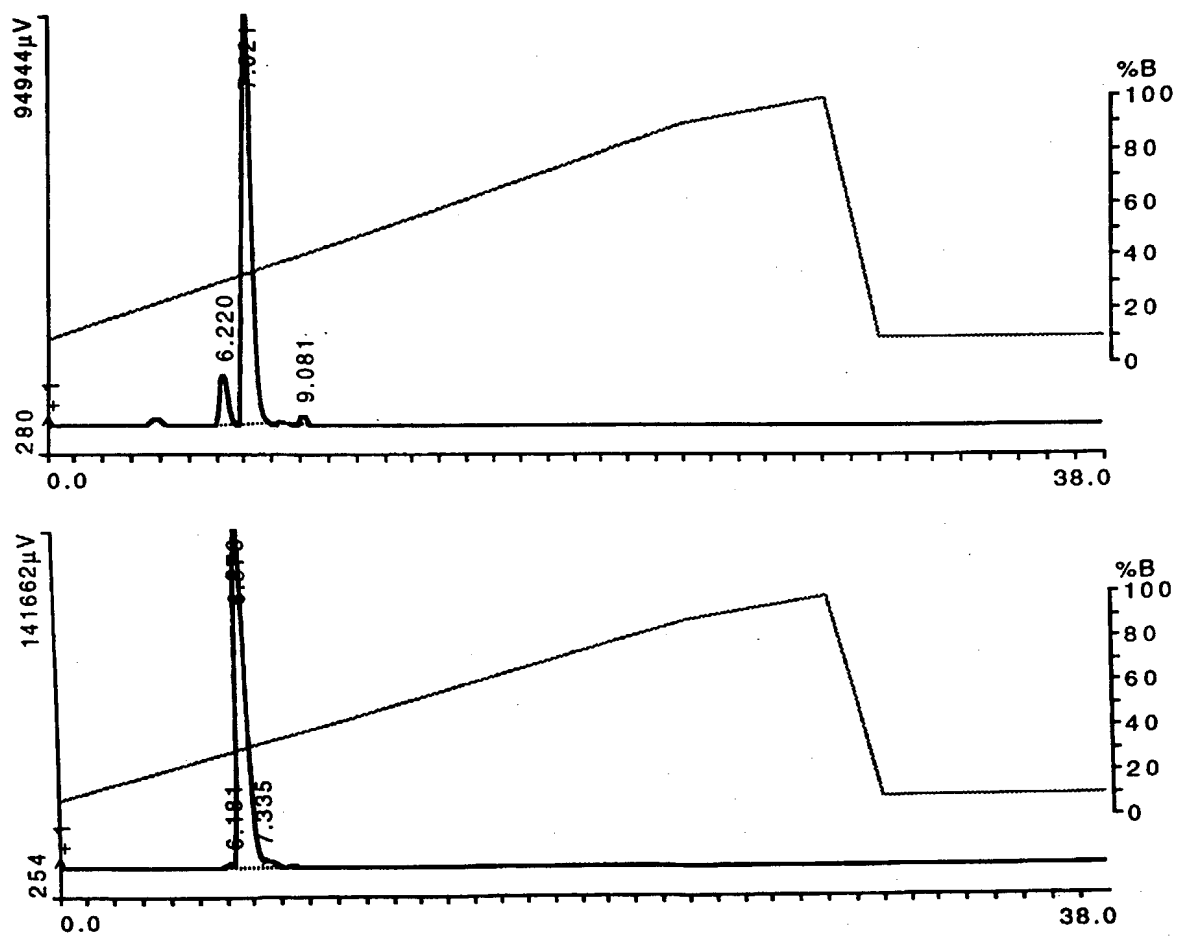


Fig. 2

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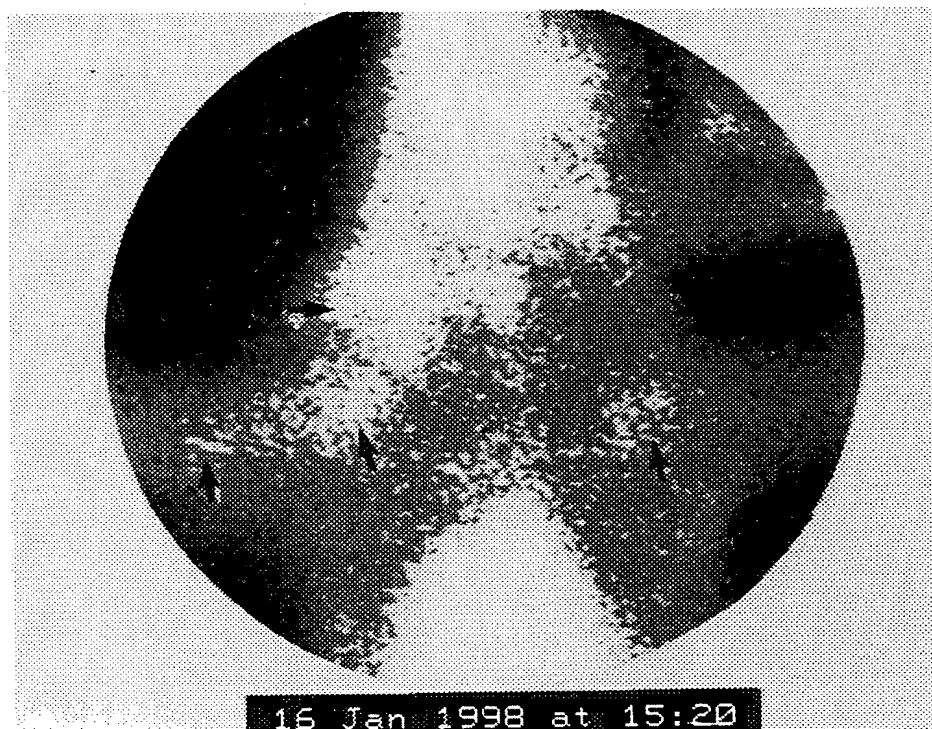
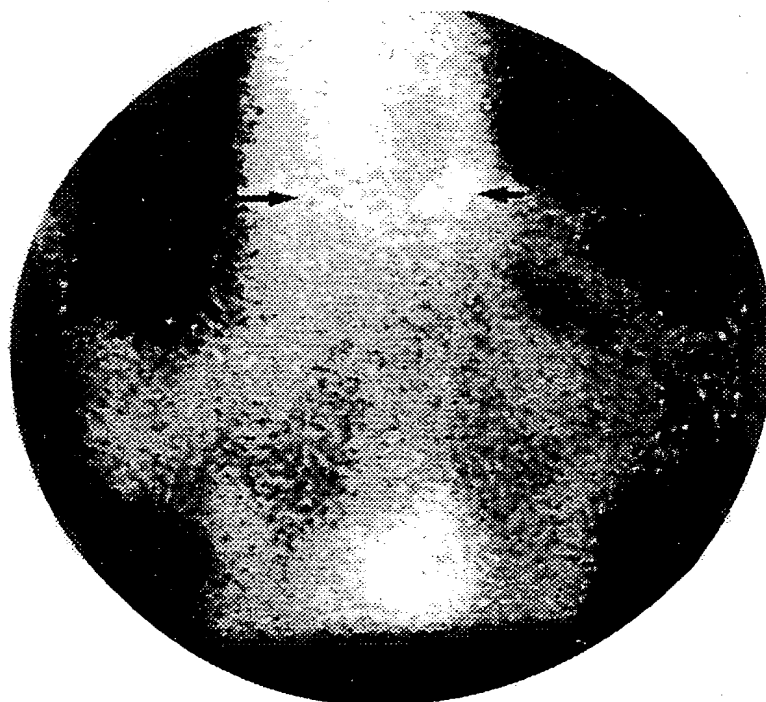


FIG. 3



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FIG. 5



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	<b>First Named Inventor</b>	THAKUR, Matthew
	<b>COMPLETE IF KNOWN</b>	
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<b>Examiner Name</b>		

**As a below named inventor, I hereby declare that:**

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**IMAGING WITH TC-99M LABELED FIBRIN-ALPHA-CHAIN PEPTIDE**

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☒ was filed on (MM/DD/YYYY) **08/17/1999** as United States Application Number or PCT International (if applicable).

Application Number **PCT/US99/19011** and was amended on (MM/DD/YYYY) \_\_\_\_\_

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
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60/096,803	08/17/1998	

[Page 1 of 2]

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NAME OF SOLE OR FIRST INVENTOR: Matthew Thakur

☐ A petition has been filed for this unsigned inventor

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☐ A petition has been filed for this unsigned inventor

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Signature

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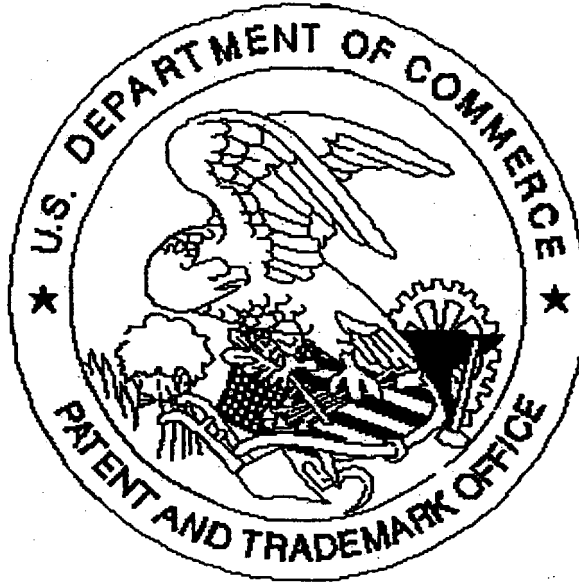
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